UNIVERSIDADE DE LISBOA

Faculdade de Medicina de Lisboa

Instituto de Medicina Molecular

The role of alpha-synuclein phosphorylation in synucleinopathies

Elisa Basso

Tese orientada por:

Prof. Doutor Tiago Fleming Outeiro

Doctoral degree in Biomedical Science, specialization in Neuroscience

Doutoramento em Ciencias Biomédica, Especialidade em Neurociencias

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Abstract

Alpha-synuclein (aSyn) is a pre-synaptic protein linked to Parkinson's disease (PD) both by genetic and pathological evidence. The gene encoding for aSyn was the first to be associated with familial forms of PD. In concert, aSyn was identified as the main component of Lewy Bodies (LBs), one of the pathological hallmarks of PD along with the loss of dopaminergic neurons from the *substantia nigra*. The discovery that the same protein –aSyn- and its aggregation propensity were involved in the pathogenesis of both sporadic and genetic PD cases propelled the aSyn research field. Remarkably, further findings characterize a range of diseases with parkinsonian features and presenting aSyn aggregates, the so-called synucleinopathies. These include amongst others PD, Parkinsons's disease with dementia, dementia with Lewy Bodies (LBs), and Multiple System Athrophy (MSA). Nonetheless, the exact mechanisms responsible for aSyn aggregation and toxicity still remain unknown. Try to understand the molecular pathways behind aSyn misfolding properties is crucial to shed light into the neurodegeneration process, and in the search for therapeutic treatments that may alleviate the social burden of PD and related diseases.

This thesis focuses on the study of the role of aSyn phosphorylation, a post-translational modification (PTM) that was shown to be essential in modulating aSyn function, aggregation and toxicity. aSyn is indeed phosphorylated on serine 129 (Ser-129) in physiological conditions, phosphorylation that goes awry during the pathogenesis of the disease resulting in 90% of aSyn phosphorylated in the LBs of brain from patients and transgenic animals models of PD. The importance of this modification became soon specific since antibodies used against this residue were adopted as common staining procedure for LBs structures. Recently, other phosphorylation sites have received intense attention; in particular tyrosine 125 (Tyr-125) phosphorylation levels were shown to be significantly reduced in diseased brains.

Despite the great effort to discover the kinases mediating aSyn phosphorylation; the contribution of this modification to aSyn toxicity and aggregation remains elusive. This is primarily due to the usage of several different methodological approaches, the difficulty to study *in vivo* such a rapid and reversible modification and the absence of phosphorylation mutants' forms that can properly mimic the effect of this PTM.

For these reasons, the purpose of this thesis was to investigate the pathways involved in aSyn phosphorylation using a simple, effective and well-established model for neurodegenerative diseases: the yeast *Saccharomyces cerevisiae*. This model has been extensively used to identify several pathways involved in PD and to generate a consistent model for aSyn aggregation that mimics the disease one. Therefore, we took advantage of the power of yeast to investigate the effects of aSyn phosphorylation on Ser-129 co-expressing human aSyn and

known kinases able to phosphorylate it; namely the Polo-like kinases family (PLKs). We studied aSyn inclusions formation and toxicity and we then proceeded to validate the results in established mammalian cell model of PD. Our approach demonstrated a unique role for one of the member of the kinases family -PLK2- in aSyn inclusion formation. We showed that PLK2 expression and aSyn phosphorylation on Ser-129 are both required for aSyn inclusion formation.

The role of aSyn Tyr-125 phosphorylation was then studied in an established oligodendroglial model of MSA. We demonstrated that this modification can prevent aSyn aggregation propensity, but only when phosphorylation on Ser-129 occurred. We then identified novel tyrosine kinases inhibitors that may be involved in the disease, but further experimental data will elucidate these potential targets.

Furthermore, using yeast we implemented a functional screening and characterized novel kinases, but most importantly, new pathways involved in aSyn pathobiology. ATG1, a kinase involved in autophagy, represented one of the major modulator of aSyn aggregation and toxicity in yeast; however this novel player requires further validation in mammalian systems to establish its potential for therapeutic purposes.

Altogether, these results provide novel insights and implications for the function of aSyn phosphorylation in PD: a co-operative role for serine and tyrosine residue embodies a novel clue to unravel aSyn misfolding behavior. Our data represent a unique opportunity to tackle one side of aSyn phosphorylation function ad further validate the importance of this PTM in aSyn pathobiology.

Resumo

A alfa-sinucleina (aSyn) é uma proteína sináptica que está associada à doença de Parkinson (DP) segundo evidências genéticas e patológicas. O gene da aSyn foi o primeiro a ser associado a casos familiares de DP e a proteína que é codificada foi a primeira a ser identificada e como maioritariamente presente nos corpos de Lewy (CL), uma das características patológicas da doença. A descoberta de que a aSyn e a sua propensão para agregar estão envolvidas não só na patogénese dos casos esporádicos como dos casos genéticos estimulou a investigação nesta área. Estudos posteriores identificaram uma série de doenças que apresentam várias características parkinsónicas, incluindo a presença de agregados de aSyn, e que por isso foram designadas sinucleinopatias. Neste grupo de doenças inclui-se a doença de Parkinson com demência, demência com Corpos de Lewy e Atrofia de Múltiplos Sistemas (AMS). No entanto, os mecanismos específicos responsáveis pela agregação e toxicidade da aSyn ainda estão por desvendar. É crucial entender as vias moleculares responsáveis que levam à perda de conformação da aSyn para aprofundar o conhecimento sobre o processo de neurodegeneração e encontrar novas terapias que possam atenuar o peso sócio-económico da DP e doenças relacionadas.

Esta tese foca-se no estudo do papel da fosforilação da aSyn, uma modificação pós-traducional essencial na modulação da função da aSyn, na sua agregação e toxicidade. Em condições fisiológicas apenas 4% da aSyn é fosforilada na serina 129 (Ser-129), enquanto que na DP 90% da aSyn está fosforilada e deposita-se nos CL dos cérebros dos doentes, um mecanismo reprodutível em modelos de animais transgénicos da DP. A importância desta modificação tornou-se específica dado que têm sido usados como procedimento comum anticorpos contra este resíduo para corar os CL. Recentemente, outros locais de fosforilação têm sido alvo de estudo, em particular a tirosina 125 (Tir-125), cujos níveis de fosforilação estão particularmente reduzidos nos cérebros de doentes.

Apesar do grande esforço para descobrir as cinases que fosforilam a aSyn, a contribuição desta modificação para a sua agregação e toxicidade é ainda desconhecida por várias razões: a utilização de métodos experimentais diferentes, o facto de ser uma modificação difícil de estudar *in vivo* dada a rapidez e reversibilidade da fosforilação, e porque não existem mutantes que consigam mimetizar convenientemente o efeito desta modificação.

Por estas razões, o objectivo desta tese é estudar as potenciais vias envolvidas na fosforilação da aSyn, usando um modelo simples, eficaz e bem estabelecido para doenças neurodegenerativas: a levedura *Saccharomyces cerevisiae*. Este modelo tem sido usado com sucesso na identificação de várias vias envolvidas na DP, tais como a disfunção mitocondrial e do proteassoma e, surpreendentemente a formação de inclusões de aSyn que mimetizam as inclusões observadas na doença. Tirando partido deste modelo caracterizámos o efeito de

cinases conhecidas na fosforilação da Ser-129, nomeadamente da família das cinases tipo-Polo. Caracterizámos a formação de inclusões de aSyn e a sua toxicidade em levedura, e posteriormente validámos os resultados em modelos celulares de mamíferos de DP. Demonstrámos um papel único da PLK2, um dos membros da família das cinases tipo Polo, quanto à formação de inclusões de aSyn. Verificámos que a expressão de PLK2 e a fosforilação de aSyn são necessários para a formação de inclusões de aSyn.

O papel da fosforilação da Tir-125 foi estudado num modelo celular já estabelecido de AMS em oligodendrócitos. Nós demonstrámos que esta modificação pode prevenir a propensão da aSyn para agregar, mas apenas quando a fosforilação na Ser-129 ocorre simultaneamente. Posteriormente identificámos novos inibidores de cinases de tirosinas que podem estar envolvidas na doença, mas mais estudos são necessários para identificar estes potenciais alvos.

Além disso, implementámos um screening funcional nas leveduras e caracterizámos novas cinases e novas vias envolvidas na patobiologia da aSyn. A cinase com maior efeito na modulação da agregação e toxicidade da aSyn foi a Atg1 que está envolvida na autofagia. É necessário validar o papel desta cinase em sistemas de mamíferos para estabelecer o seu potencial terapêutico.

Em resumo, estes resultados proporcionam novos conhecimentos e implicações para a função da fosforilação da aSyn na DP: um papel conjunto da fosforilação na serina e tirosina que aponta uma nova pista para desvendar a perda de conformação da aSyn. Os nossos resultados representam uma oportunidade única para estudar a função da fosforilação e por outro lado validar a importância desta modificação pos-traducional na patobiologia da aSyn.

Abbreviations

6-OHDA – 6-Hydroxydopamine
AD – Alzheimer's disease
aSyn – Alpha-synuclein
BAC – Bacterial artificial chromosome
BBB – Blood brain barrier
BiFC – Bimolecular fluorescent complementation assay
CHIP – Carboxyl terminus of Hsp70-interacting protein
CKs – Casein kinases
CMA – Chaperone mediated autophagy
CSF – Cerebro-spinal fluid
CSPalpha – Cysteine string protein alpha
DA – Dopamine
DLB – Dementia with Lewy Bodies
GBA – Glucocerebrosidase
GCIs – Glial cytoplasmic inclusions
GFP – Green fluorescent protein
GNIs – Glial nuclear inclusions
GRKs – G-coupled-receptor kinases
HDAC – Histone deacetylase
HSPs – Heat shock proteins
KO – Knock-out
LBs – Lewy Bodies
LNs – Lewy Neurites
LRRK2 – Leucine-rich repeat kinase 2
MAO-B – Monoamine oxidase B
MAPT – Microtubule associated protein Tau
MPPP – 1-methyl-4-phenyl-4-proprionoxy-piperidine
MSA – Multiple System Atrophy
MT – Microtubule
NBIA – Neurodegeneration with brain iron accumulation type I
NCIs – Neuronal cytoplasmic inclusions
NNIs – Neuronal nuclear inclusions
ORF – Open reading frame
PD – Parkinson's disease
PDD – Parkinson's disease with dementia
PLD – Phospholipase D

PLKs – Polo-like kinases

PRKN – Parkin

Prp – Prion

PTMs – Post-translational modifications

PUFAs – Polyunsaturated fatty acids

rAAV – Recombinant adeno-associated virus

ROS – Reactive oxygen species

S129A – SA

S129D – SD

Ser-129 – serine 129

SIAH – Seven in absentia homologue

TH – Tyrosine hydroxylase

TOR – Target of rapamycin

Tyr-125 – tyrosine 125

TPPP – Tubulin polymerization-promoting protein

1. Introduction

1.1. Synucleinopathies

In 1997 alpha-synuclein (aSyn) was identified as one of the main proteins present in Lewy Bodies (LB), one of the pathological hallmarks of Parkinson's disease (PD) [1]. Abnormal depositions of fibrillar aSyn characterized a group of diverse disorders collectively called synucleinopathies [2]. The most common aSyn disorders include PD, Parkinsons's disease with dementia (PDD), dementia with Lewy Bodies (DLB), Multiple System Athrophy (MSA) and Neurodegeneration with brain iron accumulation type I (NBIA) [3, 4].

Although synucleinopathies are characterized by the deposition of filamentous aSyn aggregates, what permits distinguishing among these disorders is the localization of aSyn depositions and their ultrastructure [5, 6]. The three main aSyn pathological depositions found in these disorders are: LBs, Lewy neurites (LNs) and glial cytoplasmic inclusions (GCIs) (Fig .1).

LBs mainly deposed in the substantia nigra of the brain, but as the disease progresses, they are also found in other areas such as the limbic system, the olfactory bulb and the neocortex. Some studies proposed that LBs pathology starts in the lower brainstem and olfactory bulb with the *substantia nigra* only affected in the middle stage of the disease, suggesting a staging progressive disease pattern with aSyn pathobiology sequentially spreading throughout the brain: the so-called Braak stages of the disease [7, 8]. Inclusion bodies have a globular/spherical structure in the cytoplasm and perinuclear compartment (LBs), and spindle-like in the cellular processes (Lewy neurites) (Fig. 1). Ultrastructurally, LBs form eosinophilic inclusions, between 5-25 μm in size, composed of a halo of straight fibrils and a dense granular core [5]. LBs mainly consist of aSyn phosphorylated on Ser-129 [1, 9], but numerous others proteins were described to deposit in LBs [10], such as ubiquitin [11], neurofilaments [12] and synphilin-1 [13]. Remarkably, LBs are present only in surviving neurons, thus their formation may represent a protective mechanism used by neuronal cells to disarmed harmful misfolded proteins [14].

Glial cytoplasmic inclusions (GCIs) can have triangular, sickle, half-moon, oval, or conical shape. Immunostaining analysis revealed a major protein component represented by aSyn. As for LBs aSyn is phosphorylated on Ser-129, and several other proteins co-deposited with it, such as ubiquitin, tubulin, and synphilin-1[15]. Ultrastructurally, GCIs consist of randomly arranged, tightly packed, granule-coated filaments, similar to the LBs ones, which are 10-20 μm in diameter. GCIs are predominately found in oligodendrocytes with few neuronal inclusions that can be easily differentiated from LBs due to their intra-nuclear localization [15] (Fig. 1). GCIs are widespread in the nervous system, although a higher density can be seen in motor cortical area, caudate nucleus, putamen, globus pallidus, cerebellar white matter and in the olfactory

bulbs. aSyn deposits are also found in another set of disorders: sporadic and familial Alzheimer's disease (AD), Down's syndrome, taupathies such as Guam parkinsonian–dementia complex, Gaucher disease and other lysosomal storage disorders and motor neuron diseases [6]. In these disorders aSyn pathobiology is considered secondary to the main pathological process. To date, some patients can present the same clinical features characterizing synucleinopathies but without aSyn deposition.

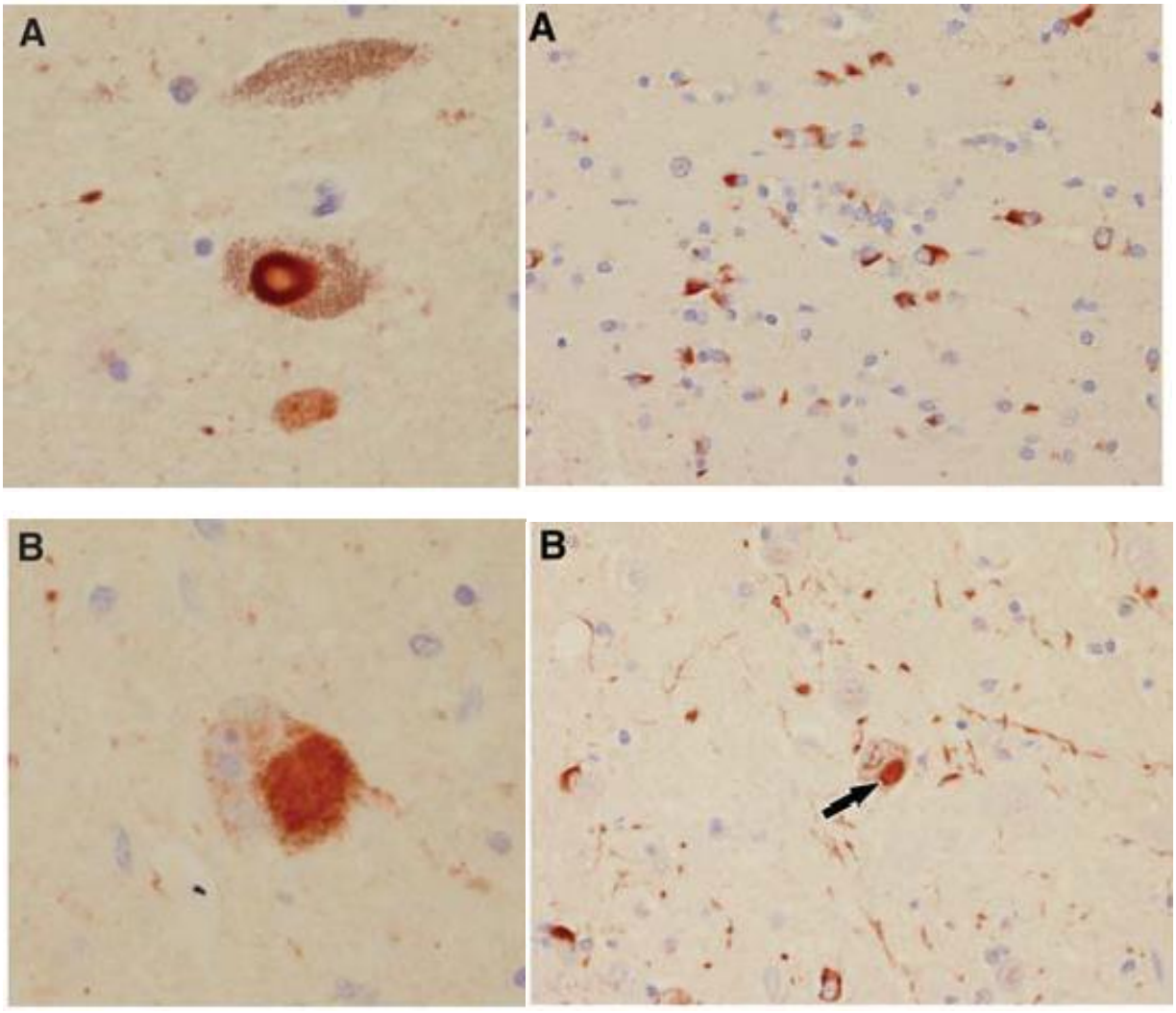

Figure 1. Microscopic findings in PD (on the left) and MSA (on the right) with aSyn immunohistochemistry. On the left panel: **A.** typical brainstem Lewy Body (LB), **B.** staining of a cortical Lewy Body (LB). On the right panel: **A.** Glial cytoplasmic inclusions (GCIs) in the putamen, **B.** neuronal cytoplasmic inclusion (NCI) in pontine nuclei (arrow). Adapted from [16].

1.2. Parkinson's disease

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder characterized by degeneration of dopaminergic neurons from the *substantia nigra* in the brain

and affecting approximately 1% of the population older than 60 years [17]. The majority of PD cases are sporadic, while only about 5% of them are known to have dominantly or recessively inherited forms that arise from specific genes. Clinically patients display bradykinesia (slowness of the movement), rigidity, resting tremor and postural instability (Fig. 2). Two are the main pathological hallmarks of the disease: intracellular proteinaceous inclusions named LBs and loss of dopaminergic neurons in the *substantia nigra pars compacta* (Fig. 2) [18]. LBs are characterized by the deposit of misfolded proteins and they are mainly composed of aSyn and ubiquitin. The biochemical nature of these deposits remained unknown until 1997 when aSyn was found to be their main component [1]. The original idea came from a previous study, where a genetic mutation in aSyn gene (SNCA) caused a familiar form of the disease [19]. These studies indicated for the first time that the same protein might be involved in both the sporadic and genetic cases of PD [1, 5, 20].

The symptoms appear in chronological order, with non-motor signs manifesting prior to motor ones. Subtle neuropsychological problems can be visible early in the course of PD, but there are not commonly assessed by clinicians. They involved problem-solving strategies, attention capacities and decision making. They manifest in difficulties with everyday activities such as organizing medications and paying bills. Memory deficits are mainly detected with further progression of the disease [21]. Autonomic dysfunctions are then described: orthostatic hypotension, constipation, insomnia, urinary frequency and urgency and sweating abnormalities [22]. Particularly, olfactory deficits may represent an early sign of PD, although research findings are not consistent [23, 24].

Neuropsychiatric symptoms are prevalent in the late stages of the disease. The most common observed are depression, anxiety, apathy and irritability, with the majority of patients displaying at least one of these symptoms [25]. Motor dysfunction manifest only after extensive dopaminergic neuron loss, suggesting the presence of a compensatory mechanism in the early stages of the disease that can counteract the progressive neurodegeneration [26]. An attributable cause for PD is still unknown but both environmental and genetics factors are believed to contribute to its pathogenesis. In PD sporadic cases several environmental risk factors have been identified: exposure to heavy metals such as manganese, copper, pesticides or brain injury might increase the risk to develop PD [17, 27, 28]. A specific case referred back to 1982, when several drug users experienced Parkinsonism symptoms while using a drug: 1-methyl-4-phenyl-4-proprionoxy-piperidine (MPPP). A side product, MPTP, derived by its synthesis was shown to be converted into MPP+ by dopaminergic neurons using endogenous enzymes: monoamine oxidase-B (MAO-B) [29]. This bio-product was able to impair the complex I of the mitochondrial chain, generating reactive oxygen species (ROS), implicated in PD pathogenesis [30].

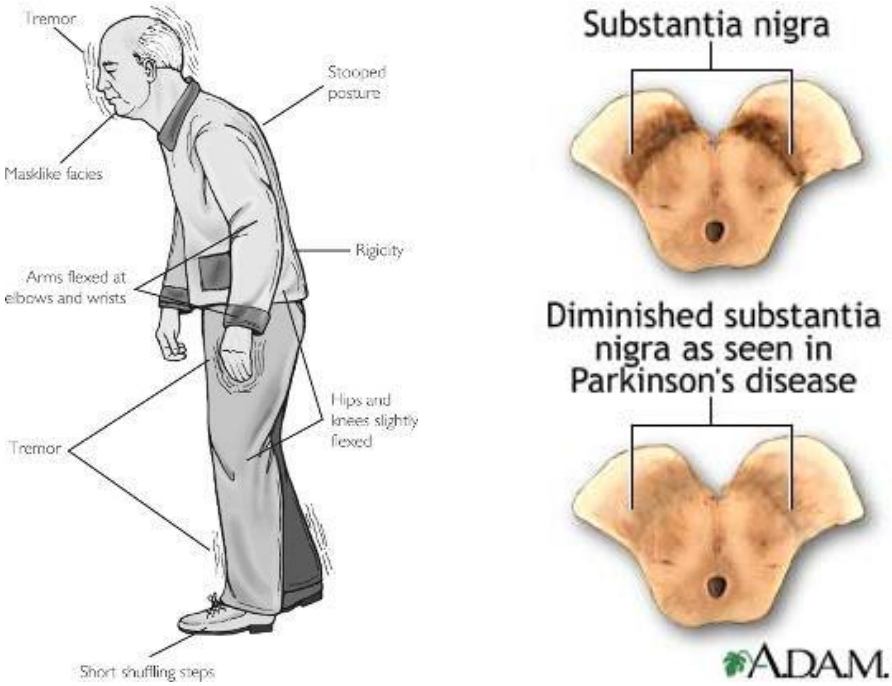

Figure 2. Clinical and pathological features of Parkinson's disease. On the left panel the major clinical manifestations in PD patients. On the right panel the selective death of dopaminergic neurons in the *substantia nigra* of PD patients (www.adam.com).

1.2.1. Genetics of Parkinson's disease

It is firmly believed that PD is determined by both multiple genetic and environmental factors; nonetheless numerous gene mutations have been correlated to autosomal dominant or recessive PD cases, counting for 5% of the total [31, 32]. Two of the genes responsible for autosomal dominant inherited cases of PD are the SNCA gene encoding aSyn [19] and Leucine-rich repeat kinase 2 (LRRK2) [33]. Autosomal recessive cases involved genes for PRKN (Parkin) [34], PINK-1[35] and DJ-1 [36] (for a complete list of genes associated with familial forms of PD see Table 1). Remarkably, all genetic forms present aSyn pathobiology; except for some cases carrying PRKN mutation and rarely LRRK2 mutation. Investigators also defined polymorphisms as risk factor for PD: carriers of a single Glucocerebrosidase (GBA) mutant allele have five time higher risk for PD [37] as well as at the SNCA gene promoter (detailed in paragraph 1.2.2). Recently, genome-wide studies have propelled the discovery of novel genes associated with PD; amongst them data showed consistent association with SNCA and microtubule-associated protein Tau (MAPT) loci [38, 39].

Table 1. Genes linked with familial forms of PD. (AD=autosomal dominat; AR= autosomal recessive; UR=unknown relevance).

Locus	Gene	Protein	Inheritance
PARK1/PARK4	SNCA	Alpha-synuclein	AD
PARK2	PARK2	Parkin	AR
PARK3	SPR(?)	(?)	AD (not validated)
PARK6	PINK1	Pten-induced kinase 1	AR
PARK7	PARK7	DJ1	AR
PARK8	LRRK2	Leucine-rich repeat kinase 2	AD
PARK9	ATP13A2	ATPase type 13A2	AR
PARK10	PARK10	(?)	UR
PARK11	GIGYF2 (?)	GRB10 interacting GYF protein 2	AR
PARK12	PARK12	(?)	UR
PARK13	Omi/HTRA2	HtrA serine peptidase 2	UR
PARK14	PLA2G6	Phospholipase A2, group VI	AR
PARK15	FBX07	F-box protein 7	AR
PARK16	PARK16	(?)	UR
PARK17	VPS35	Vacuolar protein sorting 35 Homolog	AD
PARK18	EIF4G1	Eukaryotic translation initiation factor 4 gamma, 1	AD (not validated)

aSyn (SNCA) was the first identified gene to cause dominantly inherited PD when, in 1996, a point mutation leading to the substitution A53T was described in Italian and Greek families. Subsequently, two other point mutations were discovered: A30P [40] and E46K [41]. Moreover, duplication and triplication of the gene locus were reported [42-44], with 50% increase in gene dosage for duplication generating idiopathic PD, while 100% increase producing a more severe phenotype with dementia [45]. aSyn protein expression correlated with the number of gene copies, demonstrating a link between aSyn expression levels, disease progression and severity [46]. Polymorphisms in the promoter region of SNCA gene were also identified [47]. An imperfect dinucleotide repeat called REP1 presents allele-length variability and shows association of individuals with 263 base pair allele-length with higher risk for PD. Interestingly, this polymorphism increases three times aSyn expression levels [48]. aSyn coding exons, particularly 5 and 6, demonstrated also genetic variability correlated with augmented predisposition for PD [49].

1.2.2. Pathophysiology of Parkinson's disease

The primary neuropathological deficit in PD is the loss of dopaminergic neurons in the *substantia nigra pars compacta* portion of the basal ganglia accompanied by LBs formation –inclusions enriched in aSyn and ubiquitin- in surviving neurons. The classic depigmentation of the basal ganglia area, visible in post-mortem PD brain, correlates with depletion of the neurotransmitter dopamine in the putamen (corpum striatum), primary site of *substantia nigra* neuronal projections. In a healthy individual dopamine is released from *substantia nigra* pre-synaptic terminals to the extracellular space where it will bind and activate two main dopamine transporters called D1 and D2. Neurodegeneration of the *substantia nigra* neurons impaired the dopamine release, decreased the nervous input to the striatum region, ultimately leading to the motor manifestations characteristic of PD. Remarkably, the neuronal changes occurring in PD, are also found in other brain systems such as serotonergic and cholinergic ones, as well as in the cortex, olfactory bulbs, and the autonomic nervous system.

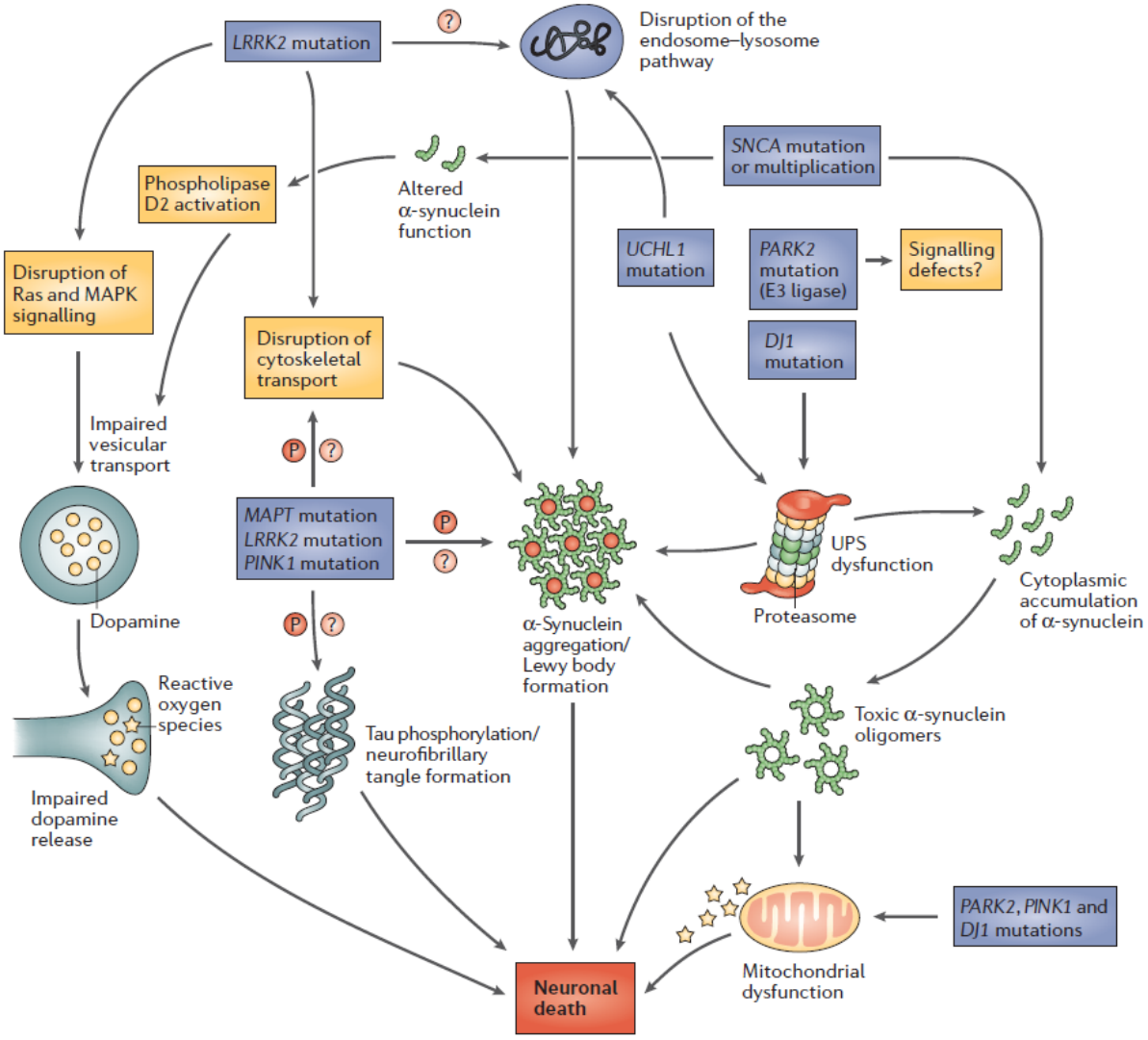

Figure 3. Intracellular pathways implicated in Parkinson's disease pathogenesis. Several factors may play a role in aSyn pathobiology: missense mutations and genomic multiplications of SNCA (the gene encoding aSyn), oxidative stress and C-terminal phosphorylation. All these factors have the capacity of increasing aSyn levels and/or promoting aSyn oligomerization and fibrils formation. The cell can respond rapidly and degraded misfolded aSyn by ubiquitin–proteasome system (UPS) and/or endosomal–lysosomal pathways. Gene mutations that may alter the activity of the UPS such as parkin and UCH-L1 can lead to neuronal toxicity. On the other side formation of aSyn oligomers, not effectively cleared, can promote their accumulation and induced toxicity by inhibiting the proteasome. aSyn genetic mutations can instead impair its vesicular binding, altering the vesicle trafficking. Similarly, aSyn can disrupt dopamine production (inhibition of tyrosine hydroxylase), packaging and vesicle dynamics, thus impairing dopamine release and promoting its accumulation in the cytoplasm ultimately generating ROS. Parkin and DJ-1 mutants can alter the proteasome function impairing aSyn clearance. Furthermore, proteasome function requiring ATP synthesis by mitochondria, mutation of PTEN-induced kinase 1 (PINK1), DJ-1 and parkin can compromise normal mitochondrial function resulting in early-onset Parkinsonism. Leucine-rich repeat kinase 2 (LRRK2) instead can play a role in cellular trafficking and intracellular signaling modulating aSyn aggregation state [50].

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It is generally believed that aSyn promotes the neurodegenerative process through a toxic gain-of-function. Triggering events involved: aSyn gene duplication/triplication or promoter polymorphisms that lead to increase protein levels [51]; gene mutations or oxidative stress that promotes aSyn misfolding behavior with formation of toxic oligomeric species [52, 53]; and cell degradation system malfunctioning, which enhances aSyn accumulation and ultimately LBs deposition [54] .

The effects produced by this toxic gain-of-function are expressed mainly at the synapses where neurotransmitters release is decreased. The SNARE protein complex (the machinery for vesicle release) is re-distributed and synaptic vesicles' recycling is inhibited [55-58]. It is believed that aSyn oligomers play an important role in this scenario, they may be able to generate membrane pores that can alters the calcium voltage-gated channels and disrupt calcium homeostasis [59-61] and/or induce leakage of dopamine at vesicles membranes, which is known to contribute to the production of ROS [62].

Oxidative stress in particular, can be triggered by several factors: mitochondrial aSyn over-expression and/or mutation can disrupt the complex I of the respiratory chain [63-65]; aSyn oligomers can induce mitochondria malfunction or morphological changes [66-68]; aSyn gene mutations or genes know to be associated with PD, such as DJ-1 [69, 70] can alter mitochondria morphology and stimulate mitochondrial autophagy; or environmental mitochondrial toxins (a known risk factor) can induce mitochondria dysfunction.

Furthermore, mutations in parkin and PINK1 can cause mitochondrial deficit and accumulation of ROS and dopamine metabolites.

A crucial factor in aSyn mediated toxicity -discovered in yeast- is the ER-to-Golgi trafficking impairment, comprising in particular Rab1, a protein involved in this transition [71]. The delay in the ER to Golgi transition arises from aSyn re-distribution at the SNARE complex [72], corroborating aSyn function and interaction with the synaptic vesicular system [73].

The formation of aSyn aggregates can be modulated by other gene mutations also associated with PD, such as LRRK2. LRRK2 co-expression with aSyn promotes aggregate formation, phosphorylation, and aSyn release into the extracellular media [74].

aSyn genetic mutants are also able to induce proteasome dysfunction in several cell cultures and *in vivo* systems [75-78], and it is thought that oligomeric aSyn species exert this effect [79, 80]. aSyn mutants can also impair chaperone mediated autophagy (CMA) [81-83], although this is still controversial. However, impairment of the degradation system could reinforce the formation of aSyn oligomeric species, aggregates formation and LBs formation.

aSyn can interact with chaperones protein such as: Hsp27, Hsp70, and Hsp90 [84]. Overexpression of Hsp27 can reduce aSyn mediated toxicity; Hsp70 was shown to inhibit aSyn fibrils binding to aSyn oligomers [85]; and carboxyl terminus of Hsp70-interacting protein (CHIP) can co-localize with aSyn in LB and decrease aSyn fibrillization [86]. Furthermore, CHIP can ubiquitinate aSyn [87] and direct it for degradation via the proteasome and the lysosome pathways [86].

A recent finding has generated great attention within the PD research field. aSyn, mostly considered an intracytoplasmic protein, it was detected in plasma, saliva and cerebro-spinal fluid (CSF) [88, 89], offering the evidence for aSyn potential secretion. It is not clear how aSyn secretion occurs, but it seems to take place through a non-classical secretory pathway. In SH-SY5Ycells aSyn can be released via exosomal vesicles whose function is dependent on calcium influx [90]. Secretion of both aSyn monomers and fibrils was also observed in cell cultures as a result of proteasome impairment or mitochondrial dysfunction [89]. aSyn release through exosomes was further confirmed in SH-SY5Y cells that present, interestingly, lysosomal dysfunction. This perturbation also produced an increase in aSyn cell to cell transmission and formation of insoluble aggregates [91]. Remarkably, extracellular secreted aSyn, especially oligomeric forms, can influence the surrounding environment leading to cell death [90, 92]. aSyn can be uptaken by neighboring cells via endocytosis, here aSyn aggregates, but not monomers, are then directed to the lysososmal compartment for degradation, reflecting a possible protective mechanism [93]. Conversely, other studies demonstrated that aSyn oligomeric species, after being uptaken, can seed fibrillization of

endogenous aSyn [60, 94-97]. Significant *in vivo* studies validated aSyn cell to cell transmission and spreading hypothesis: progenitor cells implanted in the hippocampus of aSyn transgenic mice were able to incorporate aSyn [98, 99]; and fetal dopaminergic grafts in humans showed LB pathology years after implantation [100 101]. A recent study using WT mice has demonstrated that a single striatal injection of aSyn fibrils produced aSyn intracellular transmission throughout the brain, reducing dopamine levels and generating motor symptoms PD-like [102]. Remarkably, LB-like inclusions with aSyn highly phosphorylated were detected and a spreading mechanism dependent on neuronal connectivity was demonstrated.

This phenomenon represents a potential explanation of aSyn propagation, according to Braak's staging hypothesis [8] and the possibility that aSyn may indeed act as a prion-like protein. aSyn staining in a series of PD patients and age-matched controls suggested that Lewy pathology evolves in a sequential and recognizable manner, starting in the olfactory system, peripheral autonomic nervous system, and dorsal motor nucleus of the vagus; then proceeding to the dopaminergic neurons of the *substantia nigra* only in the middle stage of the disease and only at later stage to the cortex layers [7] (Fig 4). These sequential processes firmly address the possibility that Lewy pathology can spread from affected to unaffected regions in a prion-like manner.

It is clear that many different factors can play a prominent role in aSyn toxicity, particularly which one initiates the pathological cascade; nonetheless each cellular process needs to be properly evaluated to unravel aSyn abberant behavior.

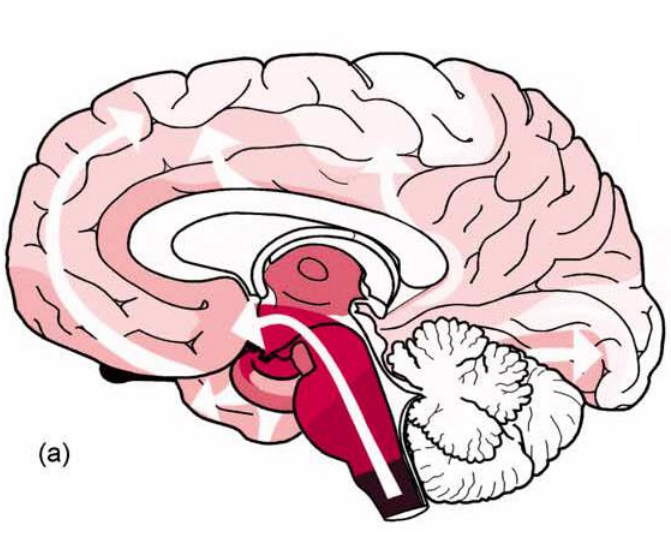


Figure 4. Distribution and progression of Lewy Body pathology according to [7, 103]. The gradual decrease in red shading intensity represents the topographical expansion of the lesions during the course of the disease. Pathology initially occurs in the dorsal motor nucleus and frequently in the anterior olfactory nucleus, then expands gradually into related areas as the brain stem (white arrows) taking an upward direction (white arrows).

1.2.3. Animal models of Parkinson's disease

Animal models are a necessary tool in the study of PD pathogenesis and progression, since research on neurodegenerative diseases requires the presence of a complex nervous system to unravel the affected biological processes. However, an animal model that fully recapitulates all the key features of the disease has still to be developed. The ideal model should be age-dependent, progressive and accompanied by dopaminergic loss [104], in addition to present motor symptoms, neurodegeneration dopamine reversible and LBs and LNs [105].

1.2.3.1. Neurotoxin models

1.2.3.1.1. 6-Hydroxydopamine (6-OHDA)

6-OHDA is a hydroxylated analogous of dopamine (DA) that uses DA transporter to reach the cytosol where it is oxidized, generates ROS and ultimately leads to oxidative stress [106, 107]. Here, it also inhibits the complex I and IV of the mitochondrial respiratory chain [108, 109].

As a well-established model of PD, it is injected stereotactically in the rat brain [110], because it cannot pass the blood-brain-barrier (BBB). Unilateral injection is preferred because bilateral injection often results in adipsia, aphagia, and death.

Several studies inject 6-OHDA into the striatum to see retrograde degeneration, because the progressive and less extensive lesion is more relevant to PD, and furthermore, synaptic terminals have been shown to die prior to *subtantia nigra* neurons [111]. 6-OHDA treatment produces the loss of more than 80% of nigrostriatal neurons [109, 112] and motor impairment [113, 114]. Notably, it has been shown to produce non-motor symptoms of PD, including cognitive and gastrointestinal dysfunction [115, 116]. The major drawbacks are the absence of LB inclusions and no phenotype age-dependecy.

The 6-OHDA model does not involve aSyn pathobiology, although it was shown to interact with aSyn [117], therefore it is primarily used as preclinical model to assess pharmacological therapies [118, 119].

1.2.3.1.2. MPTP

The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model dates back to the early 1980s when several Californian drug users showed symptoms similar to PD [29, 120]. These patients had self-administered synthetic desmethylprodine (MPPP) contaminated with MPTP [29]. Successful treatment with L-DOPA and post- mortem analysis demonstrating loss of nigrostratial connections, improved the knowledge on PD and the molecular mechanisms behind the disease [120, 121].

MPTP is lipophilic thus can easily cross the BBB. It is subsequently uptaken by astrocytes and metabolized to MPP+ by monoamine oxidase-B (MAO-B). MPP+ is a toxic molecule uptaken by dopaminergic neurons through dopamine transporter [122, 123] where interacts with the complex I of the mitochondria respiratory chain generating ROS [124]. MPTP primarily damages the nigrostriatal dopaminergic neurons [122, 125, 126].

The toxin is administered to animals by stereotactical, intravenous or intraperitoneal injection [109]. Non-human primates treated with MPTP showed aSyn positive LB-like structures and presented similar clinical, pathological and pharmacological response as idiopathic PD [127, 128]. LBs formation remains controversial, although described in monkeys, it is not present in mice unless chronically exposed over 30 days at low doses, while rats are resistant to the toxin. MPTP monkey model is mainly used to distinguish behavioral and symptomatic components of PD, whereas mouse to study the neuronal death process, and as an initial screening tool to test potential treatments for PD [129]. Anyway, MPTP represent the most used toxin based animal model of PD due to its ability to produce PD-like effects in humans and non-human primates, its reproducible nigrostriatal system lesions, and its easy routes of administration.

1.2.3.1.3. Paraquat and Rotenone

Once MPTP was described as a potential molecule to model PD pathophysiology, similar compounds started to be screened. The herbicide paraquat (N,N'-dimethyl-4-4'-bipiridinium) was identified in 1985 [130]; paraquat seems able to pass the BBB using the neutral amino acid transporter [131, 132], accumulates in the brain age-dependently, but it is still unknown how enters the dopaminergic neurons.

Toxicity is mediated by generation of superoxide [133], although the damage induced in the dopaminergic system has not been consistently observed [134, 135]. Systemic injection can be used to induce motor deficits and loss of dopaminergic neurons in a dose- and age-dependent manner [136-138]. Its treatment generates aSyn aggregates that resemble LBs in PD [139, 140]. Although epidemiological studies suggested an increased risk for PD after paraquat exposure [141-143], the lack of significant effect on striatal dopamine depletion limits the use of this model for neuroprotection studies.

Rotenone is a widely used pesticide, highly lipophilic that can cross the BBB and act on the complex I of the mitochondrial respiratory chain. The rotenone model was only implemented in 2000 developing a chronic low-dose treatment [144]. Infused continuously, rotenone produced selective nigrostriatal neurodegeneration and aSyn cytoplasmic inclusions. This study suggests that a chronic low-dose treatment may be required to produce LBs, a feature difficult to reproduce in other toxin-based animal models [145]. Rotenone can also be inhaled, subcutaneously injected or orally delivered [146]. Remarkably, in rats, motor dysfunctions and dopaminergic cell loss were reversed by L-DOPA treatment [144, 147, 148] and toxicity affected other neuronal systems as described in PD [149]. The rotenone infusion model, despite its positive features, has not been generally adopted; this is mainly due to the inability to reproduce consistently the parkinsonian phenotype [150-152].

1.2.3.2. Genetic models

1.2.3.2.1. Animal models

The number of genetic models based on PD associated genes is enormous and far from the purpose of this thesis, therefore only the principal findings will be summarized.

aSyn genetic models are much influenced by the promoter used to induce aSyn expression [104]. Under Thy-1 promoter, aSyn is highly expressed in the *substantia nigra*; in some mice motor impairment is present as well as inclusion bodies, the latter not resembling LBs [153]. The Prion (Prp) promoter induced instead a widespread aSyn expression but, mostly, not consistent with PD pathology [154]. Only the transgenic for aSyn A53T genetic mutation showed aSyn aggregation and age-dependent neurodegeneration [155, 156]. Tyrosine hydroxylase promoter (TH) directs aSyn expression exclusively in the dopaminergic neurons, but only double mutants for A53T and A30P aSyn genetic mutants showed loss of dopaminergic neurons,

motor impairment and decreased in dopamine levels age-dependent without protein inclusions [157]. One reason of the failure of these models may reside in Syn fibrillization propensity; indeed little amounts of mouse aSyn are able to inhibit human aSyn fibrillizzation *in vitro* [158]. A conditional transgenic mouse was recently generated using aSyn under a tetracycline promoter, although it failed to show aSyn aggregation, displayed dopaminergic cell loss and progressive motor impairment [159]. Interestingly, mice knocked-out (KO) for aSyn did not show alterations in the dopaminergic neurons development or maintenance, however they present a reduction in striatal dopamine and an attenuation of dopamine-dependent motor response to amphetamine [160]. Remarkably, these mice are also resistant to MPTP and other mitochondrial toxins [125, 161].

Rat models of lentiviruses and adeno-associated viruses are also used to model PD pathology. This method allows stereotactical injection of aSyn constructs directly in the *substantia nigra*, even in older animals. Anyway, they require proper handling and they may have reproducibility issues. Rats overexpressing aSyn WT or mutants showed selective loss of nigral dopaminergic neurons [162] that in adult animals was accompanied by aSyn positive inclusions [162-164]. Recently, human aSyn bacterial artificial chromosome constructs (BAC) have been used in rats, here aSyn expression showed prominent coversion to C-terminally truncated form. In old animals aSyn was converted into insoluble fibrils, resembling PD ones, and the animals suffered severe loss of the dopaminergic projections [165].

In *Drosophila* models, over-expression of aSyn WT, A53T and A30P mutations develop motor deficits, loss of dopaminergic neurons age-dependent and inclusions formation [166]. *C. elegans* models over-expressing aSyn showed dopaminergic neuron loss but no LB-like inclusions [167, 168]. The effect of aSyn mutations on dopaminergic neurons did not show major differences compared to aSyn WT. Although flies and nematode models lack of endogenous aSyn, their use allowed the discovery of numerous genes and pharmacological modifiers of aSyn toxicity. Both fly, nematode and mouse models demonstrated an increase in aSyn toxicity in presence of rotenone and 6-OHDA [169], corroborating environmental factors as risk for PD.

Saccharomyces cerevisiae - another powerful model to study aSyn pathobiology - for the purpose of this thesis will be described in details in section 1.6.

LRRK2 mutations are associated with dominant inherited form of PD: overexpression of LRRK2 in *Drosophila* and *C. elegans* leads to degeneration of dopaminergic neurons [170-172]. Mice KO for LRRK2 did not display any toxicity related

to neuronal dopaminergic development and maintenance [173], while several LRRK2 mouse models showed only low levels of neurodegeneration [174].

Mutations to parkin, DJ1 and PINK1 cause autosomal recessive forms of PD. *Drosophila* models displayed age-dependent loss of dopaminergic neurons and locomotors abnormalities, whereas the mice models failed to replicate many key features of PD. KO rodent models of these genes did not show nigrostriatal degeneration, or cytoplasmic inclusions that resembles PD cases, except for PINK1 KO mice showing reduced dopamine release in the striatum [175]. However, recently KO parkin in old mice generated neurodegeneration in the *substantia nigra* [176].

1.2.3.2.2. *In vitro* models

The number of *in vitro* models of PD is broad, therefore, for the purpose of this thesis, only the principal mammalian cell models will be described.

The expression of solely aSyn in several cell models does not induce cytoplasmic inclusions or strong cytotoxicity, thus other methods started to be employed, such as protein overexpression or toxin-based. Neuronal cells over-expressing aSyn were shown to form cytoplasmic inclusions and presented mitochondrial deficits [177]. Human neuroblastoma cells expressing aSyn A53T or A30P mutants, exposed to hydrogen peroxide or the neurotoxin MPP+ were more vulnerable to oxidative stress, particularly the A53T one [178]; similar results were obtained using menadione-induced oxidative stress [179]. In another model, neuroblastoma cells over-expressing aSyn WT, A53T and A30P mutants showed increased ROS production, more pronounced in the mutants. Remarkably, exposure to dopamine decreased cell viability, phenomenon further enhanced in the mutants [180]. Likewise, only accumulation of aSyn in cultured dopaminergic neurons leaded to dopamine dependent apoptosis and ROS production [181].

aSyn A53T or A30P inducible expression in differentiated PC12 showed increased sensitivity to apoptotic cell death (while treated with proteasome inhibitors), mitochondrial depolarization and elevation of caspase 3 and 9 [76].

Further methods took advantage of known aSyn binding protein detected in LBs. Co-expression of the protein synphilin-1 and aSyn promotes the formation of cytoplasmic inclusions [13, 182]. The fusion of green fluorescent protein (GFP) to the C-terminal of aSyn was shown to undergo truncation within the GFP domain and to induce aSyn aggregation [183, 184]; this aggreagation propensity was further enhanced by co-expressing synphilin-1 (Fig. 5).

The bimolecular fluorescent complementation assay (BiFC) has demonstrated great impact in the study of aSyn oligomers formation. This assay involves the fusion of two non-fluorescent fragments of GFP to the proteins of interest. In case an interaction occurs between the two proteins of interest, the reporter fragments can combine together and reconstitute the activity of GFP [185]. Stabilization of aSyn oligomers via BiFC in human neuroglioma cell line, increased intracellular toxicity, which could only be rescued reducing oligomers formation [186] (Fig. 5).

Other cellular models relied on aSyn extracellular uptake as seed for the fibrillization of the intracellular aSyn [96, 97, 187, 188]. Unfortunately cell culture studies showed some discrepancies on the effect of aSyn expression and/or mutations on citotoxicity; in some, aSyn expression made the cells more sensitive to cellular stress [189, 190], while in others was shown to be protective [191, 192]. These discrepancies probably arise from the use of different cell lines, different promoters or protein tags [193].

Although the scientific community is still looking for the perfect PD model, the studies hereby described have demonstrated to be essential to identify the molecular mechanisms (yeast, worms and flies), the biological pathways (cellular models) and the behavioral features (animal models) involved in PD pathogenesis and aSyn pathobiology.

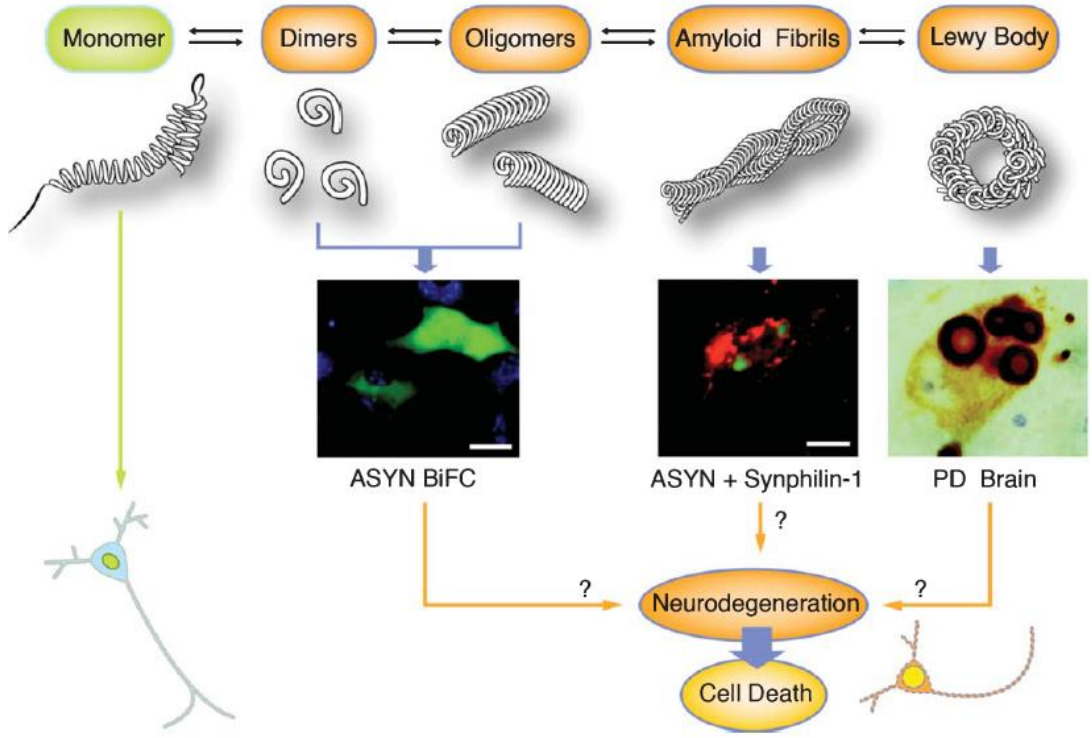


Figure 5. Schematic representation of aSyn aggregation models. Misfolded aSyn monomers may promote the formation of dimers and oligomers that, if failed to be degraded by the intracellular protein degradation machinery, can seed aggregation of other aSyn monomers leading to fibrils formation and ultimately to LBs proteinaceous inclusions.

Several assays are used to characterize which species can be formed at cellular levels: the bimolecular fluorescence complementation (BiFC) assay, which depends on formation of a fluorescent complex, allows visualization of aSyn dimers and oligomers (green); aggregates formation can instead be modelled co-expressing aSyn and synphillin-1 (red), which positively react for ThioflavinS staining (green) [194].

1.3. Multiple Systems Atrophy

The term Multiple Systems Atrophy (MSA) was initially used to describe three different disorders that had as common feature neuronal atrophy: olivopontocerebellar atrophy [195], striatonigral degeneration [196] and Shy-Drager syndrome [197, 198]. MSA was included in the synucleinopathies group when Papp and colleagues described that glial cytoplasmic inclusions (GCIs) –one of the neuropathological hallmarks of these disorders- contained aSyn as main component [199].

MSA is a progressive, sporadic disease, with adult onset (between 55 and 58 years old). Clinical hallmarks comprise variable combination of parkinsonism, cerebellar ataxia, autonomic dysfunction and/or corticospinal dysfunction [200]. MSA motor phenotype is primarily divided into patients with predominant parkinsonism (MSA-P) or cerebellar ataxia (MSA-C) [201]. The heterogeneity of clinical features increases the chances of wrong diagnosis as well as the absence of specific biomarkers, thus a definitive validation necessitates of post-mortem histopathological analysis.

The main pathological features comprise: neuronal loss, gliosis, myelin pallor and severe axonal degeneration. In addition, between 10 to 32% of MSA patients suffer from cognitive dysfunction [202]. Reactive astrocytes, containing swollen and intense GCIs, and activated microglia are common histological findings in MSA white matter, with astrogliosis paralleling the severity of neurodegeneration [203, 204] (Fig. 6).

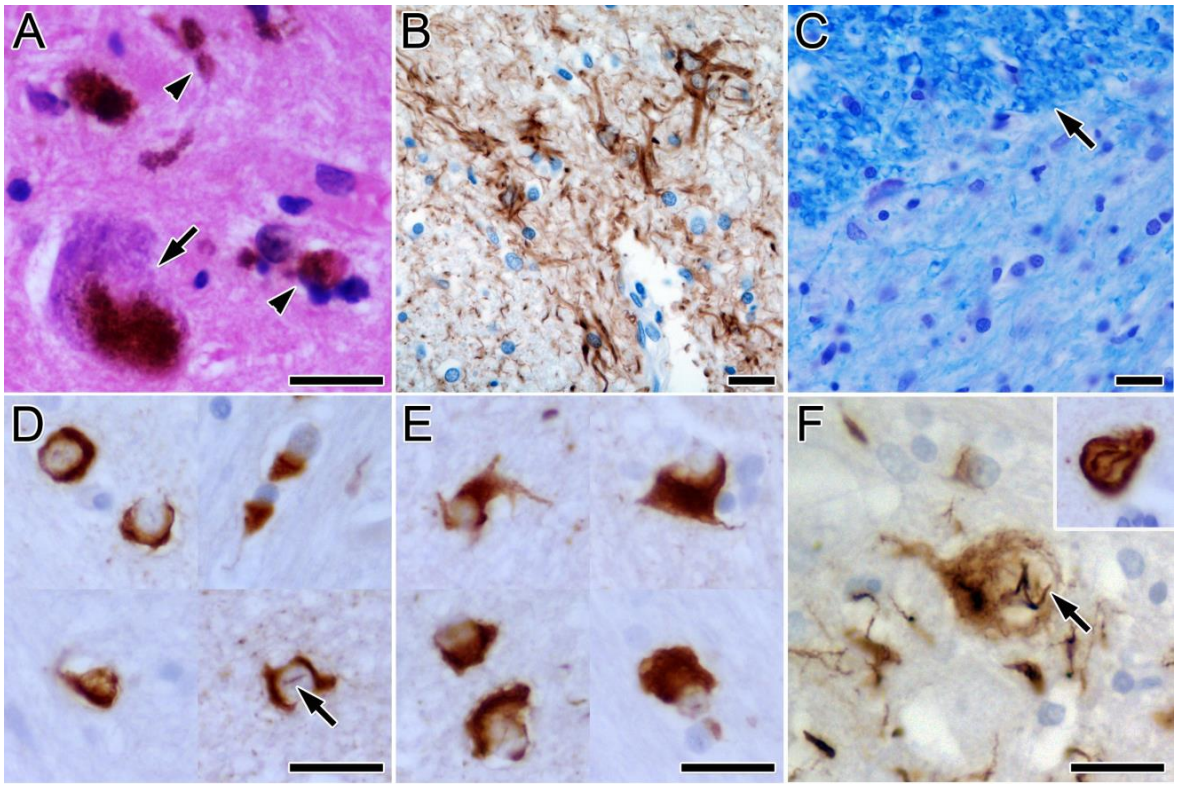


Figure 6. Common histological features of Multiple System Atrophy (MSA). A. Neuronal loss (arrowheads) **B.** gliosis (hypertrophic astrocytes) **C.** myelin pallor (blue staining). **D-E** Putamen immunohistochemistry using antibodies specific to aSyn shows glial cytoplasmic inclusions (GCIs), the hallmark lesions of MSA (arrows show intra-nuclear inclusions). **F.** Intra-nuclear inclusions (arrow). [205].

The characteristic neuropathological hallmarks of MSA are cytoplasmic inclusions in oligodendroglial cells called glial cytoplasmic inclusions (GCIs). aSyn is the main component and antibodies against it represent the most specific methods to identify GCIs; anyway, other proteins are found in GCIs, namely ubiquitin and p62. GCIs have variable diameter, 5 to 20 µm, and are located close to the nucleus. They present several morphologies such as triangle, half-moon, oval, conical and flame-shape, remarkably, the nucleus of GCIs containing cells is larger than the lacking one. Ultrastructurally, GCIs are randomly arranged, loosely packed filaments coated with a dense granular material [206]. aSyn fibrils can be organized as twisted filaments (5-18 nm diameter) and straight filaments (10 nm diameter) [3], similarly to aSyn ones in PD and DLB. The widespread presence of GCIs aSyn positive classifies GCIs as unique and sufficient markers to diagnose MSA, even without a clinical history [207]. Interestingly, aSyn can accumulate in other cell types: in the nuclei of oligodendrocytes to form glial nuclear inclusions (GNIs) [199], in the cytoplasm and nuclei of neurons as neuronal cytoplasmic inclusions (NCIs) and neuronal nuclear

inclusions (NNIs) [208], and in cell processes and neuritis (Fig. 6). While GNIs are difficult to observe, NCIs and NIIs are consistently found in MSA [209].

GCIs present another common component: p25, also known as tubulin polymerization-promoting protein (TPPP) [210, 211]. p25 has a significant role in microtubules stabilization, oligodendrocytes projections, and ciliary structures[212]. A prominent function involved oligodendrocytes differentiation since p25 is expressed while the myelination process occurs [213, 214]. Remarkably, during the neurodegeneration process, p25 delocalizes from myelin to the oligodendrocytes cell soma, increasing cell body size, promoting GCIs formation and suggesting that p25 redistribution may be an early event in the generation of GCIs [215]. It was indeed shown that p25 presence within the cellular body favors aSyn aggregation, contributing to oligodendroglial dysfunction and neuronal degeneration [216]. It is still unknown the reason why aSyn is found in oligodendrocytes since it is a protein of neuronal origin; furthermore studies addressing its role in non-neuronal cells have led to contradictory results. The analysis of aSyn mRNA levels in brain regions known to be affected in MSA, resulted either invariant or down-regulated in MSA brain compared to controls [217, 218]. Differential expression of mRNA aSyn splicing variants was detected in MSA and PD brain [219, 220], while in situ hybridization for aSyn and proteolipid protein, an oligodendrocyte marker, failed to characterize aSyn expression in either MSA or control brains [221]. Conversely, rat oligodendrocytes brain cultures expressed aSyn but in a developmental manner [222]. Novel hypothesis are considering that aSyn expression and/or degradation may be up-regulated during specific steps of the neurodegenerative process or uptaken by neighboring neurons. The former hypothesis gained ground when detergent soluble aSyn was primarily found in MSA brains, while only a small amount was SDS insoluble, suggesting that altered solubility may precede GCIs formation [223].

Similarly to LBs, aSyn in GCIs is found post-translationally modified: nitration [30, 224] and phosphorylation at Ser-129 are the major modifications [9], indicating oxidative stress and aSyn aggregation as principal factors involved in MSA pathogenesis. The foremost mechanism of pathogenesis cascade predict that early aSyn overexpression and/or delocalization may influence p25 distribution from the myelin to the oligodendrocytes cell soma, generating myelin dysfunction and increasing the cell soma size [215]. This mechanism will initiate the neurodegeneration process, followed by microglia activation [225], aSyn fibrils formation and deposition in GCIs [226]. This process is further enhanced by p25 delocalization and incorporation into the same inclusions [211]. The oligodendrocytes soma gets then larger, the nucleus becomes pale and myelin degenerates [215]. Ultimately, the formation of GCIs disrupts cellular functions and leads to cell death [227]. Cell death may be responsible for aSyn aggregates release into the extracellular that may be uptaken by nearby neurons: a potential source for the formation of NCIs. Thus, persistent neuroinflammation [228],

oligodendroglial and neuronal dysfunction, and aSyn inclusions formation could altogether contribute to the neurodegenerative process and potentially spread it into other regions of the brain [229]. Although there are still missing pieces in this process, this hypothesis is contributing to investigate the potential cellular mechanisms involved in MSA pathogenesis, particularly the ones that trigger aSyn presence in the GCIs.

1.3.1. Genetics of Multiple Systems Atrophy

Genetic variations can modulate susceptibility to develop sporadic forms of neurodegenerative diseases, recent studies have suggested that genetic factors may play a role also in MSA [230]. Studies on few families suggested rare Mendelian inherited forms of MSA. In a German family with autosomal dominant inheritance, the mother and one daughter presented classical features of MSA whose diagnosis was confirmed histopathologically in the mother [231], and another family with two members affected having potential autosomal dominant inherited form [232]. Autosomal recessive inheritance was described in multiple Japanese families [233], and in UK were found ten proven cases of MSA [234]. Nonetheless, these studied failed to identify a causative gene, mainly due to the limited number of affected individuals.

To improve the identification of MSA related genes, screens were performed among genes known to be involved in other neurodegenerative diseases with overlapping pathology. aSyn gene, SNCA, was scanned with no positive results, although variation within the locus were associated with a risk of MSA [235-237]. To date, SNCA is the most robust genetic association so far identified.

The gene MAPT encoding for Tau protein, was also associated in pathological validated cases of MSA [238] and interestingly, few MSA cases present Tau localization within the GCIs [39, 239].

1.3.2. Animal models of Multiple Systems Atrophy

Animal models of MSA are based on three different approaches: toxin-based lesion of striatonigral structures; overexpression of human aSyn in oligodendrocytes; and the two methods combined [240].

Toxin-based models (6-OHDA, MPTP, quinolinic acid and 3-nitropropionic acid) failed to reproduce the characteristic pathological hallmark of MSA, GCIs, thus limiting their practice.

Transgenic mice model overexpressing aSyn under protein lipid promoter demonstrated GCIs formation and biochemical composition similar to MSA ones, although not argyrophilic. Mice also showed age-related loss of dopaminergic cells in the *substantia nigra* and subtle motor impairment [226, 240]. Mice overexpressing aSyn under 2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNP) promoter presented also GCI-like inclusions in oligodendrocytes, degraded myelin, cortical atrophy together with age-related motor impairment [241]. These models support evidence of neurodegeneration as a result of human aSyn overexpression in oligodendrocytes; nevertheless, the mild motor deficits could not qualify them as absolute model for MSA.

For these reasons researchers started to use a combined approach. 3-nitropropionic acid, a mitochondrial inhibitor was administered to the PLP-aSyn overexpressing mice [240]. This model showed GCI-like inclusion formations, neuronal loss in the striatum, and severe motor and cerebellar impairment, thus replicating many of the clinical features of MSA and suggesting that aSyn is the key element that renders oligodendroglial cells vulnerable to oxidative stress.

Remarkably, the MSA mice models described above showed consistently microgliosis [240], as described in the pathological MSA cases. A recent study using aSyn overexpression under PLP promoter indicates that microglial activation may be an early event in the pathogenesis of the disease underscoring neuronal loss [242].

1.3.3. An oligodendroglial cell model of Multiple Systems Atrophy

aSyn overexpression in glioblastoma, astrocytoma cell line or rat primary mixed glial cultures leads to the formation of intracellular fibrils. In the same models expression of a C-terminally truncated form of aSyn promotes further the formation of aSyn aggregates [243]. In oligodendroglial cells derived from primary Wistar rat brain glial cultures (OLN-93 WT) aSyn WT and A53T mutant expression can positively model MSA, inducing aSyn aggregation, increasing its insolubility and promoting oxidative stress [244]. This model was used in the study of this thesis and will receive a further description.

In rat oligodendroglial OLN WT cells co-expression of aSyn and p25 induces aSyn-dependent toxicity and progressive cellular degeneration dependent on the ability of p25 to promote aSyn aggregation [216]. p25 is primarily expressed in rat nervous tissue [214] and localizes specifically to the cytoplasm and myelin sheaths of oligodendrocytes in adult rats [245]. p25 can play a pathogenic role in MSA since can stimulates aSyn aggregation of α-syn *in vitro* [216] and it was shown to redistribute from the myelin to oligodendrolial cell soma prior to accumulation and aggregation of αSyn in GCIs [215].

The co-expression of aSyn and p25 initiates a cellular response characterized by accumulation of soluble aSyn oligomers phosphorylated at Ser-129, activation of caspase-3, retraction of the microtubules (MT) to the perinuclear region and apoptotic cell death (Fig. 7). Apoptosis involves activation of caspase 3, phosphatidylserine externalization, nuclear condensation and fragmentation. The toxicity is dependent on aSyn aggregation since treatment with amyloid aggregation inhibitors (ASI1D peptide, baicalein and CongoRed) abrogates MT retraction. Most importantly, the toxicity depends on aSyn phosphorylation on Ser-129 because the aSyn SA mutant reduced the toxicity as well as addition of serine kinase inhibitors (DMAT, DRB, and emodin). Moreover, treatment with DMAT was able to reduce aSyn soluble oligomeric high molecular weight species [246]. The same results were subsequently reproduced showing that oligodendroglial apoptotic cell death was partially rescued by sirtuin-2 inhibition [227].

Recently, it was demonstrated that the cytotoxicity caused by co-expressing aSyn and p25 relies on stimulation of the death domain receptor FAS and caspase-8 activation. Primary oligodendrocytes derived from aSyn transgenic mice express pro-apoptotic FAS receptors, which make them sensitive to FAS ligand-mediated apoptosis. These results were corroborate by an increase FAS expression found in brain extracts from MSA cases [247], suggesting FAS expression as an early hallmark of oligodendroglial degeneration.

A novel study using differentiated PC12 catecholaminergic nerve cells showed that expression of p25 is also able to induce aSyn delocalization into autophagosomes. Remarkably, p25 prevented autophagosomes fusion with lysosomes, causing an increase in aSyn secretion to the medium. aSyn secretion was shown to be dependent on autophagy, suggesting p25 involvement in aSyn extracellular release and potentially its spread during the disease process [248].

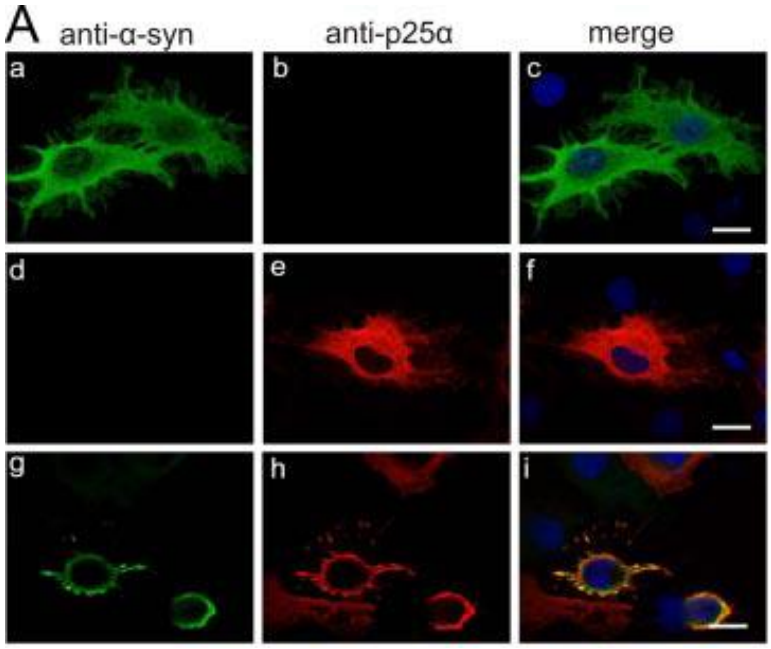


Figure 7. Retraction of cellular processes in oligodendroglial glial co-expressing aSyn and p25. OLN WT cells transiently transfected with human aSyn and empty vector (a–c), p25 and empty vector (d–f), and aSyn and p25 (g–i). The retraction of microtubule (MT) processes is selective for the co-expression of the two proteins. [246].

1.4. Alpha-synuclein

1.4.1. Alpha-synuclein structure

aSyn is a 140 amino acid protein present in the nervous system but also in other tissues, such as heart and blood. The name derived from its original localization: the nucleus and the pre-synaptic nerve terminals [249]. It was purified for the first time as core component of the amyloid deposits in Alzheimer's disease –the NAC region- subsequently identified as the aSyn protein [250]. Thanked to the first genetic mutation identified in the aSyn gene (A53T) [19] and the succeeding positive staining for aSyn within LBs inclusions [1], aSyn became the major gene (SNCA) linked to sporadic PD. These studies demonstrated for the first time that aSyn is a main component of PD inclusion bodies and, mostly, the idea that its aggregation properties may be involved in the pathogenesis of the disease.

The amino acid sequence of aSyn can divided into three regions: 1) the N-terminal region, from residue 1 to 60, characterized by six imperfect repeats of the consensus motif KTKEGV. This region is predicted to form amphipathic alpha-helices upon membrane and lipid vesicles binding [251-253]. 2) A central hydrophobic region from residue 61 to 95, called NAC, is highly hydrophobic and crucial for fibril formation [254]. 3) The C-terminal negatively

charged (rich in glutamate and aspartate residues) and with highly disorder structure (Fig. 8). aSyn is highly conserved among mammals, does not exist in invertebrates, and the C-terminal domain is the most variable in size and sequence [255]. Remarkably, the majority of vertebrates contain the A53T disease-linked mutation, whereas in primates and human an alanine is present at this position [255]. aSyn is a member of the synuclein family of proteins, which include beta and gamma-synuclein. aSyn principal difference with their family members is their lack of NAC domain and consequently their inability to form fibrils. All are mainly neuronal proteins and under physiological conditions localize preferentially to presynaptic terminals [256]. aSyn is natively unfolded due to its high hydrophilicity and negative charges at the C-terminal [257, 258]. Recently, this notion has been challenged by a study suggesting a natively tetramer conformation that result stable in aqueous solution and unable to aggregate [259]. Notably, the majority of the studies performed until now rely upon the expression of a 14 kDa aSyn protein that may not mimic adequately the true native nature of the protein; nonetheless further analysis are essential.

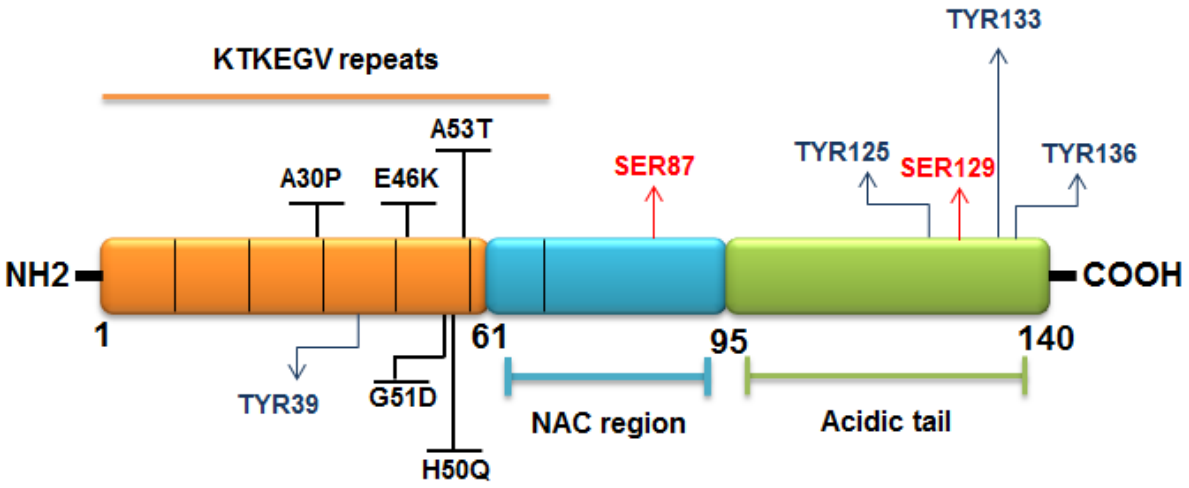


Figure 8. Biochemical structure of aSyn. A schematic representation of aSyn structure highlighting: the N-terminal region (orange), the NAC region (light blue), the C-terminal domain (green), the genetic mutations (bold) and the serine (red) and tyrosine (dark blue) sites of phosphorylation. Numbers refer to amino acid residues flanking the different regions. Ser=serine, Tyr=tyrosine. Adapted from [194].

1.4.2. Alpha-synuclein function

aSyn is an abundant protein in the nervous system, accounting for 1% of the total protein. It localizes mainly in proximity of pre-synaptic terminals and less prominently in the neuronal soma [260]. It has been also found in erythrocytes and platelets, with still unknown function

[261]. Little is known about the exact physiological function of aSyn, but numerous works contributed to understand the role of this protein in the nervous system. aSyn expression levels were initially found increased during synaptic plasticity. Evidence came from studies on zebra finch and song learning, where aSyn homologous protein expression and pre-syncaptic localization augmented in the brain regions involved in the bird song [262-265]. Subsequent work exploited null aSyn animal models and/or cellular models over-expressing aSyn. aSyn KO mice showed altered dopamine release [160], while in primary hippocampal neuronal cultures treatment with aSyn antisense oligonucleotide reduced the distal pool of synaptic vesicles [266]. Using another aSyn KO mice model, it was demonstrated that aSyn may be required for the maintenance of vesicles from the reserve pool and may thus regulate synaptic vesicle mobilization at terminals [267]. Using cells over-expressing aSyn caused a reduction in secreted catecholamines and hypothesized that aSyn may inhibit the vesicle priming step prior to exocytosis [268, 269].

Remarkably, the usage of aSyn fluorescently labeled, revealed that aSyn moved from the synaptic terminal during neuronal firing [270]. Altogether these studies underscored a prominent role for aSyn in synaptic transmission. These effects are determined by aSyn ability to bind cellular membrane and vesicles, to interfere with phosphatidic acid metabolism, and to compartmentalize resting and releasable vesicles pools [266]. Notably, aSyn can act as a chaperone to inhibit protein aggregation as for instance esterases during heat and pH stress [271-273]. Here, the C-terminal domain is responsible for its thermal stability and for its chaperone activity [274]. Consistently, in mice KO for cysteine string protein alpha (CSPalpha), which display synaptic degeneration [275], aSyn expression can alter significantly their phenotype. In this scenario, aSyn may chaperon CSPalpha in the assembly of SNARE complex [275, 276], thus controlling neurotransmitter release through the SNARE complex and function.

The role of aSyn in synaptic transmission and regulation of the vesicles pool for storage or release of neurotransmitters may play a distinct role in the pathogenesis of PD, particularly modulating dopamine production and demonstrating susceptibility towards a specific neuronal type. Remarkably, monomeric aSyn reduces the activity of Tyrosine Hydroxylase (TH), the rate-limiting enzyme for the conversion of L-DOPA; this may happen at gene expression levels or by interacting with the enzyme [277, 278]. When aSyn forms aggregates this modulation can be disrupted, increasing cytoplasmic dopamine. Furthermore, PD patients brain scan have shown decreased VMAT expression, a membrane transporter involved in dopamine storage [279], interestingly, this finding was not associated with aSyn A53T overexpression mutant, reflecting a different function from aSyn WT in dopamine storage [280]. Similarly, aSyn can bind to the dopamine transporter (DAT) and influences dopamine re-uptake into the neurons [281, 282].

1.4.3. Alpha-synuclein aggregation

aSyn is a highly dynamic protein characterized by intermediate states of fibrillization. *In vitro* studies demonstrated that aSyn generates under prolonged time of incubation, fibril-like structures considered similar to LBs inclusions [283, 284]. Initially, aSyn forms oligomeric soluble species with spherical, ring, and string-like appearances as shown by electron microscopy [285-287]. Oligomeric soluble species are difficult to detect *in vivo*, but, recent studies using native gels or size-exclusion chromatography have managed to characterize them [288-290]. Oligomeric species gradually generate structures named protofibrils and, ultimately, recruiting more monomer, they aggregate into insoluble fibrils (Fig. 9) [291] . This so-called nucleation-dependent mechanism is divided in a lag phase: oligomers structure will form; and a rapid growth phase: fibril formation via monomer addition; for then reaching a steady-state where equilibrium between monomeric and fibrillar species is formed [292] (Fig. 9).

aSyn fibrils represent the pathological state of the protein since it was demonstrated a lack of aSyn toxicity without the NAC aggregation-promoting domain [254, 293, 294], and a protection mechanism by overexpressing HSPs (chaperones that assist other proteins into their correct re-folding) [295-297]. Conversely, oligomeric species are considered the toxic ones. A severe dopaminergic loss was shown only in rats treated with aSyn mutants that form oligomers (E57K and E35K), while those that formed fibrils were less toxic [290]. Dopamine and its metabolites can also inhibit formation of mature fibrils both *in vitro* and *in vivo* [298], suggesting that dopaminergic vulnerability in PD may be linked to increase aSyn oligomers formation. Furthermore, oligomers were also found in plasma and cerebro-spinal fluid of PD patients and their levels seem to correlate with the disease state [299, 300]. Oligomeric species of aSyn WT and A53T mutant have been described in brain tissue from aSyn transgenic mice and PD patients [301], and elevated levels of soluble oligomers have also been detected in post-mortem brain extracts from DLB patients compared to control brains [302].

It is necessary to mention that some protofibrils are not toxic, the so-called “off-pathways”, while formation of larger aggregates can also engulf and disrupt the intracellular neuronal machinery, therefore the toxicity induced by oligomeric aSyn is still under debate.

Conditions influencing aSyn aggregation can be: non-specific molecular crowding [303] and protein concentration [304]. These reflect possible pathological mechanisms: multiplication of aSyn gene is known to promote oligomeric formation and mitochondria dysfunction [44, 305], and aSyn oligomers and larger aggregates seem able to block proteasome degradation [79, 80, 306]. Remarkably, aSyn mutants can promote aSyn oligomerization, but only A53T mutant can augment aSyn rate of fibrillization [283, 285, 307-309]. Further studies demonstrated that

membrane-bound aSyn has higher propensity to aggregate [310], similarly, induced oxidative stress (treatment with peroxynitrite and rotenone) can also enhance aSyn aggregation [311-313] and cell toxicity in rats model of PD [147, 314]. aSyn is able to bind metal ions modulating its oligomerization state, for instance copper-induced oligomeric specie can be cytotoxic [286], while Fe^{3+}-induced oligomers can form permeating pores at lipid-bilayers [315].

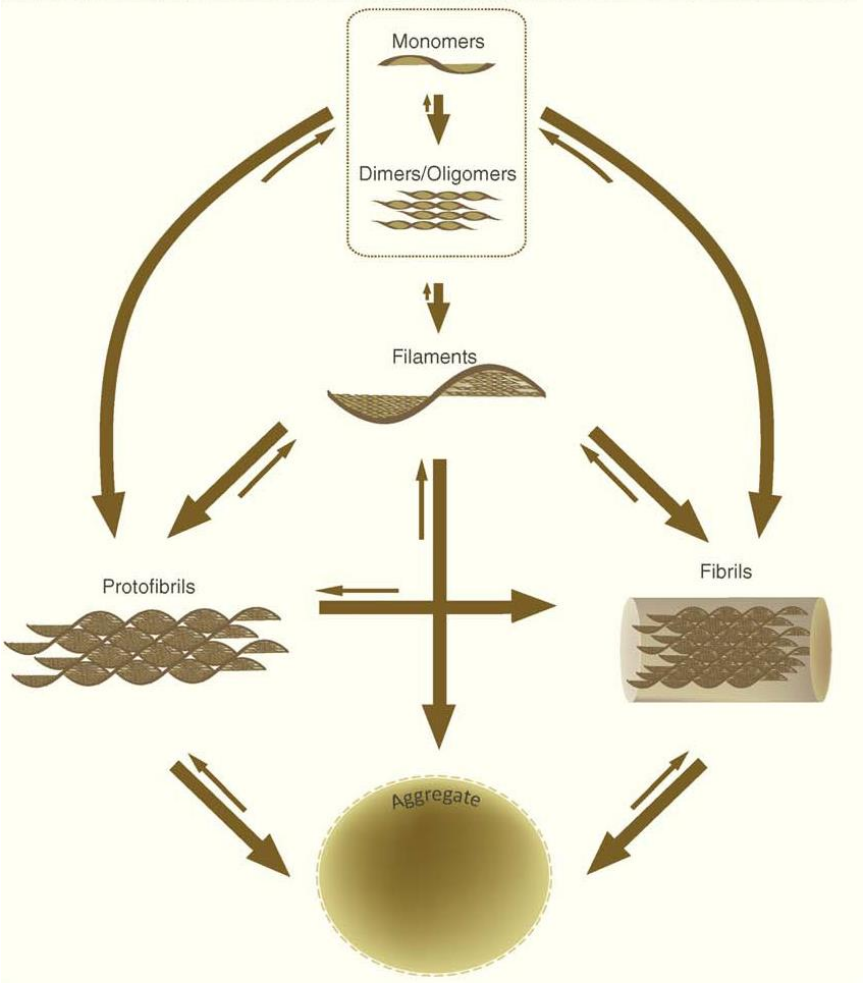


Figure 9. Nucleation of aSyn aggregates. aSyn monomers can form soluble dimers/oligomers that successively can accumulate into insoluble species (filaments, protofibrills, fibrils and aggregates). In this scenario oligomeric species seem to be more toxic than larger protein aggregates. aSyn aggregation propensity can arise from increased gene dosage, environmental factors or non-specific moleculr crowding. Adapted from [185].

1.5. Alpha-synuclein post-translational modifications

Several PTMs are known to occur in aSyn present in the LBs of PD patients and related diseases. Also, several of them have been identified in animal models of synucleinopthies. However, it is not clear yet, how these PTM affect aSyn structure and folding behavior and how they modulated the pathogenesis of the disease. Here, we will summarize the principal modifications associated with the disease and the current knowledge on their role on aSyn pathobiology.

1.5.1. Alpha-synuclein phosphorylation

Phosphorylation is the most common post-translational modification used to modulate or change a protein function. aSyn is physiological phosphorylated on Ser-129 and Ser-87 residues in cell cultures [316]. The discovery that aSyn was also phosphorylated on Ser-129 in synucleinopathies lesions propelled studies on the role of this PTM in aSyn function and misfolding behavior [9]. Immunohistochemistry analysis of synucleinopathies brain tissues found out that 90% of aSyn deposited in LBs was phosphorylated at Ser-129, compared with the physiological 4% of normal brains [317, 318]. For the first time, the role of aSyn phosphorylation on Ser-129 became prominent and, remarkably, a shared phenomenon in the spectrum of synucleinopathies. Notably, phosphorylation of aSyn at Ser-129 increases during LBs formation, that is, when aSyn was turning into an insoluble protein [319]. The same study identified truncated and ubiquitinated forms of aSyn in LBs, but at much smaller proportion compared with phosphorylated ones [319]. aSyn can indeed undergo several PTMs while deposited in the LBs; it can be phosphorylated at serine and tyrosine residues, truncated, nitrated, ubiquitinated, sumoylated, acetylated and enzymatically crossed-linked (Fig. 11).

Remarkably, aSyn phosphorylation was reproduced and correlated with PD pathology in numerous animal models: in transgenic mice over-expressing aSyn [226, 320-322], in aSyn transgenic rats [323] and flies [324, 325].

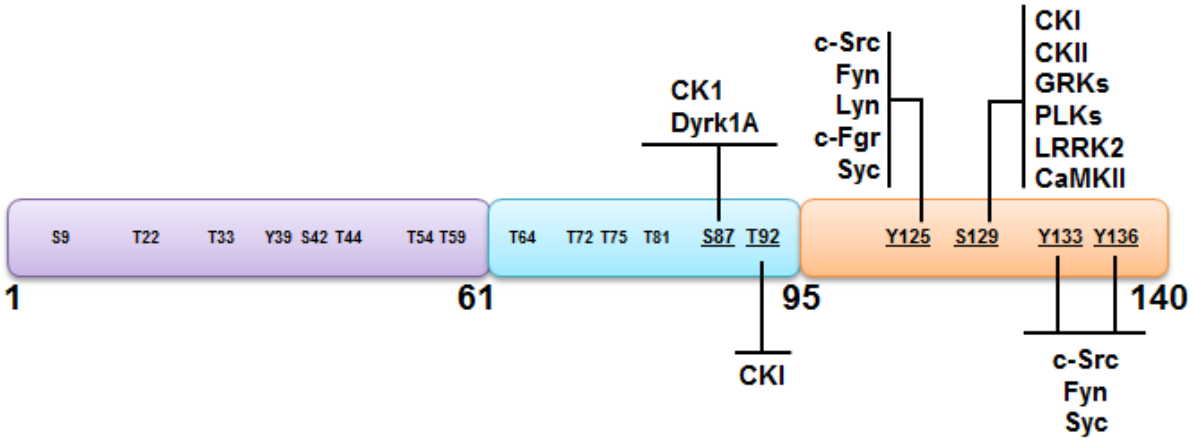


Figure 10. aSyn potential phosphorylation sites and associated kinases in humans. (S=serine, Y=tyrosine, T=threonine). Adapted from [326].

1.5.1.1. Phosphorylation at Serine residues

aSyn contains four serine residues: serine 9 and serine 42 (N-terminal domain); serine 87 (NAC region) and serine 129 (C-terminal domain); whith serine 129 residue highly conserved among species (Fig. 9). Serine 87 and serine 129 were characterized using phosphorylation mutants (aSynSA that mimics the non-phosphorylated state of the protein) in cell lines [316]; serine 129 (Ser-129) phosphorylation was identified in LBs [9]; while only in 2010 phosphorylation of aSyn at serine 87 (Ser-87) in LBs was discovered [327].

The natural kinases and phosphatases involved in aSyn serine phosphorylation/dephosphorylation remain unknown, but potential candidates have been identified *in vitro* and *in vivo*. Kinase specific for Ser-129 are G coupled-receptor kinase 1, 2, 5 and 6 (GRK) [328, 329] and the Polo-like kinases 2 and 3 (PLK) [330, 331]. Casein kinase 1 and 2 (CK) can phosphorylate aSyn both at Ser-87 and Ser-129 residues [316]. CK2 and GRK-5 were also found in LBs [332, 333] (Fig. 9). Not all the kinases have the same efficiency to phosphorylate aSyn; remarkably PLK2 and PLK3 can fully phosphorylate aSyn *in vitro* [330, 331] both in monomeric and fibrillar states [331, 334]; while in cerebral cortex of PLK2 KO mice phosphorylated aSyn was significant reduced [330]. PLK2 cellular levels seem regulated by the ubiquitin–proteasome system [335] since during synaptic stimulation proteasome inhibition leads to an increase in PLK2 levels [336]. Interestingly, PLK2 expression levels were found elevated in post-mortem brain tissue from subjects with AD and LBD [331]. Amongst phosphatases, *in vivo* studies

showed that Phosphoprotein Phosphatase 2A can dephosphorylate aSyn on Ser-129 [337], while *in vitro* studies characterized Phosphoprotein Phosphatase 2C [338].

The effect of phosphorylation on aSyn toxicity and aggregation remains still controversial; the choice of different model system and/or methodological approaches produced many discrepancies. In a transgenic *Drosophila* model, expression of aSynSD phosphorylation mutant (mimics the phosphorylated state of the protein) induced dopaminergic loss that was totally reverted when the aSynSA mutant was instead expressed. In the same model co-expression of aSyn WT and GRK2 increased aSyn Ser-129 phosphorylation levels and cell toxicity [325]. In the first recombinant adeno-associated virus (rAAV) based rat models of PD, [339] expression of S129A generate loss of *substantia nigra* dopaminergic neurons, whereas S129D reverted it. In a subsequent rat model, S129A was shown to increase dopaminergic cell loss, while S129D did not alter it [340], while in a third model it was reported no difference in dopaminergic neuronal cells expressing either aSyn WT, S129A, or S129D [341].

As alternative, *in vivo* mediated aSyn phosphorylation co-expressing in rats GRK6 kinase with aSyn genetic mutant A53T enhanced toxicity, while the S129A mutant did not display any changes compared with aSyn WT [342]. Similarly, a transgenic mouse model over-expressing aSyn with an enhanced PP2A phosphatase activity, showed a better motor performance and increased neuronal activity, but data on dopaminergic cell loss were not assessed [337]. These studied clearly exemplified the complex nature behind aSyn phosphorylation on Ser-129 and the difficulties to model such a peculiar PTM in animal models of PD. On top of this, methodological approaches may affect the results and render them difficult to compare. For instance, aSyn expression levels, the viruses serotype, and observational time points resulted different in the various models [343]; furthermore it was demonstrated that the phospho-mimicking mutant S129D does not replicate the structural properties of authentically Ser129-phosphorylated aSyn. Therefore, the results obtained with this mutant have to be considered critically [344].

The role of aSyn Ser-129 phosphorylation on aSyn aggregation is also under intense debate. *In vitro* data reported opposite results: Ser129 phosphorylated may or may not inhibit aSyn fibrillization [9, 344]. In a transgenic fly model, Ser-129 phosphorylation suppressed formation of aSyn aggregates [325] and reduction in aSyn C-terminal charge seems to favor formation of fibrillar species [345]. Further data suggested that Ser-129 phosphorylation may occur after aSyn aggregation [331, 334]. Recently, it was described that formation of aSyn insoluble species parallel an increase in soluble and lipid-associated aSyn Ser-129 phosphorylated in PD and DLB brain tissues in regions still not presenting LBs, suggesting that Ser-129 phosphorylation may occur before aSyn

aggregation [346]. In addition other data showed that induction of phosphatase 2A can reduce aSyn aggregation [337].

Others authors focused on the physiological role of aSyn phosphorylation to try to narrow which process may be principally involved in PD pathogenesis. Studies demonstrated that Ser-129 phosphorylation can be a signal for aSyn nuclear localization [321, 331, 347]. Nuclear staining was indeed confirmed in numerous model systems: in transgenic mice for A30P and A53T aSyn mutants compared with control ones [348]; in HEK cell line over-expressing aSyn and GRK5 compared with WT one [331, 347]. The consequences of this localization are unknown but studies revealed that aSyn may play a function in transcriptional regulation since nuclear translocation was shown in response to the herbicide paraquat [349], and to reduce histone acetylation [350]. Moreover, inhibitors of histone deacetylase (HDAC) rescued aSyn mediated toxicity in cell cultures and in *Drosophila* models [350].

Phosphorylation can be also a signal for protein degradation as proteasome inhibition in cell and primary neurons increased aSyn phosphorylation on Ser-129 [351, 352]. This phenomenon was shown to be ubiquitin independent [352].

aSyn can bind to numerous proteins such as TH and the dopamine transporter (DAT), modulating their function [277, 353-355]. Remarkably, some interactions can modulate its aggregation propensity: synphilin-1, a protein identified through a yeast two-hybrid screen as aSyn interacting protein, can promote aSyn aggregation and inclusion formation [13]. In yeast co-expression of aSyn and synphilin-1 accelerated inclusion formation, and, interestingly co-expression of synphilin-1 mutant R621C (a genetic association found in idiopathic PD cases, [356]) enhaced also aSyn phosphorylation at Ser-129. Furthermore, synphilin-1 inclusions co-localized with lipid droplets and endomembranes [357].

Furthermore, aSyn interaction can sequestre arachidonic acid within the SNARE complex inhibiting neuronal transmission [358], and impair the hydrolysis of phosphatidylcholine to phosphatidic acid regulated by the enzyme phospholipase D (PLD) [359, 360]. Although this latter finding was not always consistent [361], PLD is linked to neurotransmitter release [360] therefore lipids balance may be required for proper synaptic function and alteration of this mechanism may play a role in aSyn toxicity. Similarly, polyunsaturated fatty acids (PUFAs) have been shown to enhance aSyn oligomerization and toxicity [301, 362, 363].

To date, only phosphorylation at Ser-129 and Ser-87 has been linked to PD. Ser-87 is not conserved amongst species, for instance mice and rats have an asparagine in position 87 and aSyn is found phosphorylated at Ser-87 in LBs and its levels were increased in AD, MSA and PD brains [327]. Phosphorylation at Ser-87 is preferentially

found in membrane fractions and kinases CK1/2 [316] and the dual specificity tyrosine-regulated kinase Dyrk1A were associated with it [364]. Ser-87 lies within aSyn NAC region and can modulate aSyn fibrils formation; *in vitro* studies demonstrated that Ser-87 phosphorylation or using phosphorylation mutants (S87D/E) inhibits aSyn fibrillization [327, 365]. I*n vivo* findings corroborate this function in parallel with a protective mechanism toward dopaminergic cell loss [366].

1.5.1.2. Phosphorylation at Tyrosine residues

Tyrosine phosphorylation is known as a very rapid process, difficult to analyze *in vivo* and that happens rarely in proteins. aSyn contains four conserved tyrosine residues: Tyr-125, Tyr-133 and Tyr-136 in the C-terminal domain and Tyr-39 in the N-terminal domain (Fig. 7). aSyn was initially shown to be phosphorylated on Tyr-125 in cell culture only upon treatment with pervanadate, a non-specific phosphatase inhibitor [367]. Subsequent *in vitro* kinase assays confirmed Tyr-125 as main phosphorylated tyrosine residue in aSyn [368-370]. Remarkably, in a *Drosophila* model aSyn resulted phosphorylated on Tyr-125, a signal enhanced upon inhibition of phosphatases [371]. *In vitro* and cell culture studies demonstrated that Tyr-125 phosphorylation is mediated by several tyrosine kinases namely Fyn [367, 368], Pyk2/RAFTK [369], c-Src [367, 369], and c-Fgr [370]; and at the three tyrosine residues Tyr-125, Tyr-133 and Tyr-136 by Syk [370] (Fig. 10). The physiological phosphatases involved in aSyn tyrosine dephosphorylation remain unknown.

Initial studies did not reveal any association between Tyr-125 phosphorylation and aSyn pathobiology [367, 368]; however shortly after it was reported that aSyn phosphorylation at Tyr-125 was induced by hyperosmotic stress [369] or by rotenone treatment [372]. Remarkably, in the only study from human brain tissue patients, the levels of Tyr-125 phosphorylation were shown to decrease with age and with PD pathology, suggesting its association with the disease [371].

Single tyrosine residue analysis has not been properly investigated yet, but phosphorylation of Tyr-125, Tyr-133 and Tyr 136 was shown to inhibit aSyn oligomerization and inclusion formation [370]. Similar conditions were addressed in flies expressing Shark kinase, a homolog of Syk, and the same effect on aSyn oligomerization was observed [371]. Furthermore, the same study characterized the effect of triple tyrosine phosphorylation mutants to mimic the non-phosphorylated state of the protein (Y125F/Y133F/Y136F). These flies showed loss of dopaminergic neurons and

reduction in climbing activity compared with control ones. These findings suggest a protective mechanism for aSyn tyrosine phosphorylation.

Other potential roles for Tyr-125 phosphorylation may be dependent on protein-protein interactions: the phospho tyrosine 125 peptide was shown to interact more preferentially with CK1 than the phospho serine 129 peptide [373] this may indicate a potential cross-talk between the two phosphorylation sites. Phosphorylation at Tyr-125 is also able to increase aSyn binding to bivalent cations such as Cu^{2+}, Pb^{2+} and Fe^{2+}, although similar findings were also observed for Ser-129 phosphorylation [374].

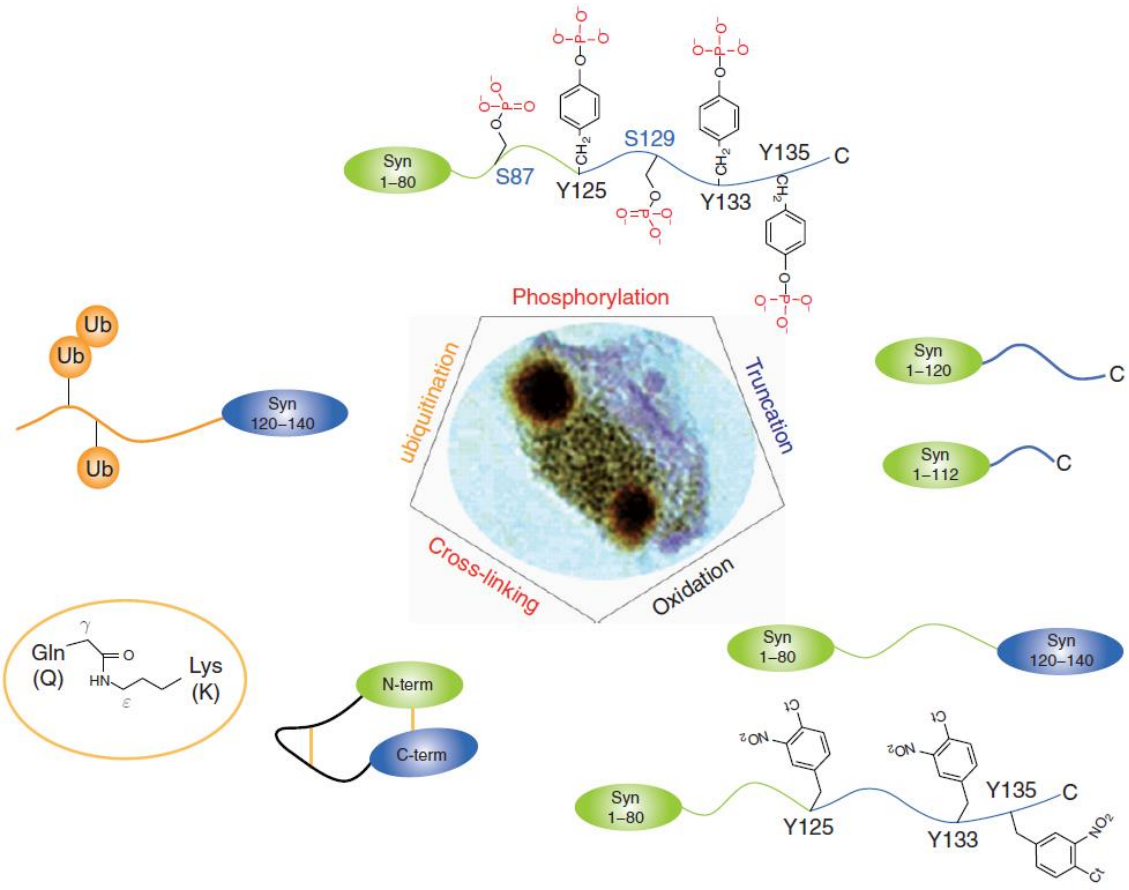


Figure 11. Major aSyn post-translational modifications found in Lewy Bodies. These include phosphorylation, ubiquitination, truncation, nitration and covalent cross-linking by tissue transglutaminase [326].

1.5.2. Alpha-synuclein ubiquitination

In 1998, ubiquitin was found to co-localize with aSyn in LBs. Subsequent biochemical analysis of PD brain insoluble fractions demonstrated that not only ubiquitin was present in the LBs but that aSyn itself was ubiquitinated [375]. aSyn can be mono- di- and to a less

extent poli-ubiquitinated at Lys-12, Lys-21 and Lys-23 as mass spectrometry studies revealed [375-377] and *in vitro* and cell culture studies confirmed [319, 378, 379]. Interestingly, some ubiquitinated forms were also phosphorylated at Ser-129 [375]. Several E3 ligases have been reported to ubiquitinate aSyn: seven in absentia homologue-1 (SIAH-1) mono-ubiquitinqted aSyn at Lysine residues 12, 21, 23 (as within LBs) but also at Lysines 10, 34, 43, and 96 [380]; Nedd4 poly-ubiquitinated aSyn through Lys-63 ubiquitin chains [381]; TRAF6 and CHIP can also ubiquitinate aSyn [87, 382].

Not all aSyn inclusions in PD patients are ubiquitin positive, suggesting that ubiquitination may not be required for aSyn aggregation [376]. Initial studies showed that aSyn ubiquitination promotes its aggregation, and cells over-expressing E3 ligase SIAH-1 presented an increase in aSyn aggregation [378]. Similarly, *in vitro* ubiquitination also increases aSyn fibrillization [380].

Protein ubiquitination is used to target proteins for degradation, in the case of aSyn studies showed dissimilar scenarios. Pharmacologically impairment of proteasome did not lead to aSyn ubiquitination [377, 383], while others described the opposite [384]; or showed that aSyn can be degraded via proteasome without ubiquitin signal [352, 385, 386]. Co-expression of aSyn and SIAH-1 did promote its degradation in presence of proteasome inhibitors [378]; however, aSyn incubation with SIAH-2 in presence of the proteasome subunit 26S was sufficient to promote its degradation [379]. Recently, it was demonstrated that Nedd4 aSyn mediated ubiquitination favors its degradation via the lysosomal-endosomal pathway [381]. These data suggest that ubiquitination may play a role in aSyn degradation, but the mechanism may depend on ubiquitination sites and/or enzymes involved.

1.5.3. Alpha-synuclein truncation

Proteomic analysis of LBs revealed also the presence of aSyn truncated forms, recognizing several aSyn proteolytic products: 1-134, 1-133, 1-126, 1-122, 1-119, 1-115 and 1-96 [319, 387, 388]. The same species were also identified in transgenic mice models of PD [155, 156, 388]; however, aSyn truncated species exist also in healthy brains. *In vitro* analysis demonstrated that aSyn truncation can promote its aggregation and can be considered an important factor into aSyn misfolding behavior [389-391]. *In vitro* assays, indeed, have so far constantly showed that aSyn C-terminal truncation promotes fibril formation and can seed aggregation of the full length protein [388]. In cell culture studies, overexpression of the truncated forms, 1-110 and 1-120, increased vulnerability to oxidative stress compared to the full-length protein [388]. *Drosophila* models expressing the truncated 1-87 form showed increased aSyn accumulation and enhanced neurotoxicity [294], however the over-expression

of aSyn truncated forms in rodent models did not so far reproduce PD pathological conditions [319]. To date, several proteases were found responsible for aSyn truncation: neurosin generates primary 1-80 residues fragments [392, 393], calpain I cleaves aSyn within the NAC region and aSyn fibrils after residue 120 [394, 395], and cathepsin D cleaves aSyn C-terminally [396]; also the 20S proteasome subunit can cleave aSyn, but only in monomeric free form [388].

1.5.4. Alpha-synuclein oxidation

ROS and nitrogen species can generate 3-nitrotyrosine aSyn, a modification identified in LBs [30]. All four aSyn tyrosine residues (Fig. 7) are found nitrated in LBs of PD brain patients [30]; oxidative stress can mediate aSyn covalent cross-linking via di-tyrosine formation; a mechanism that can stabilize aSyn oligomers *in vitro*, inhibiting aSyn fibrils formation [397], and can contribute *in vivo* to the formation of oligomeric species [398]. In rats model nitrated aSyn was shown to be toxic to dopaminergic neurons leading to motor deficit, most probably due to the action of aSyn oligomeric species [314].
All four aSyn methionine residues can be oxided *in vitro* to methionine sulfoxide. This modification can disrupt aSyn structure, blocking its propensity to form both oligomers and fibrils [399]. It was shown that the inhibition of aSyn fibrils formation is proportional to the number of methionine residue oxided [400]. Conversely, methionine sulfoxides can bind to metal ions such as Zn^{2+}, Pb^{2+} and Al^{3+} and promote aSyn fibrillization [401].
Dopamine itself can be higly oxidized, a status that binds aSyn inducing the formation of SDS insoluble aSyn oligomers formation [402].

1.5.5. Alpha-synuclein acetylation

aSyn is also ubiquitously acetylated at its N-terminal metionine residues in LBs [319]. This PTM seem unable to affect aSyn aggregation propensity or subcellular localization [403-406], although inhibition of sirtuin 2, a mammalian HDAC that increases acetylation, was associated with neuroprotection in various models of synucleinopathy [407, 408].

1.5.6. Alpha-synuclein sumoylation

Recently aSyn was shown to be sumoylated at Lys-96 and Lys-102. The protein responsible for this PTM are SUMO1, and to a less extent SUMO2 [409]. Small-ubiquitin-like modifiers (SUMO) are proteins that possess structural similarities to ubiquitin. Although the

significance of aSyn sumoylation has to be explored, recent studies have suggested that aSyn sumoylation may prevent its aggregation and that even small amounts of sumoylated proteins can inhibit aSyn aggregation [410].

1.5.7. Alpha-synuclein transglutaminase cross-linking

aSyn is highly cross-linked between lysine and glutamine residues in dopaminergic neurons of PD and the extent correlated with the disease progression [411]. Glutamine 79, 99 and 109 were found as residues acceptors for Lysine 60 residue donor at the N-terminal of aSyn [412]. Cross-linking inhibited aSyn fibrils formation and promoted oligomer unstructured formation [413] that are unable to disrupt lipid bilayers. However, the effect of cross-linking on aSyn was shown to be dependent on the position of the cross-link; with the majority generating aSyn non-toxic oligomers.

1.6. *S. cerevisiae* as a model for neurodegenerative diseases

Many of the significative advances in our understanding of the pathogenic mechanisms underlying PD have exploited yeast as model system; the usefulness of this organism in respect of higher eukaryotes resides in the conserved homology of genes and pathways in such distant evolutionary context. This knowledge was fundamental to discriminate which pathways were involved in diseases context and permitted a powerful approach to the field of neurodegenerative research.

Saccharomyces cerevisiae was the first eukaryote to be completely sequenced in 1996 [414]. 6,600 open reading frames (ORFs) (genome sequence that encode proteins) are characterized and 80% functionally described [415, 416]. Remarkably, 60% of the yeast genes present a human homologous or a domain that is functionally correlated with a human one [417]. Homology to yeast genes has often applied to identify novel genes in higher eukaryotes, and among the several biological processed involved in the neurodegenerative diseases, the majority result conserved in yeast, such as transcriptional factors, protein targeting and secretion, cytoskeletal dynamics and protein quality control. Together with its genetic manipulability and short generation time [418], these features rendered yeast the ideal model for high-throughput screening [419]. Yeast phenotypes resulting from genetic, environmental or chemical causes can be analyzed by genome-wide screens, transcriptional profiling, proteomic and lipidomic

analysis. A collaborative yeast scientific community guarantees that the data produced can be screened against public available databases such as genome-wide deletion, reduced expression or overexpression libraries [420]. Such libraries include collections of plasmids for manipulating the expression of every ORF of the yeast genome – as the one used in our study. Yeast does have limitations such the lack of interplay between immune and inflammatory responses, and of a nervous system; furthermore mammalian cells have several cellular specializations without homology in yeast. Despite critics, yeast has been an utterly informative system to identify novel genes and environmental connection in neurodegenerative disorders, and to identify new therapeutic targets [421].

The basic pathways altered in neurodegenerative diseases are highly conserved between yeast and human species; therefore, the fundamental molecular events involved in these disorders can be studied in yeast [73]. Protein quality control was the first process analyzed in yeast and involved in neurodegenerative diseases. Protein misfolding that result into generation of proteinceous inclusions is one of the key pathological hallmarks of these disorders. The first recognized misfolding disease is caused by prions: infectious protein particles transmissible from cell to cell, in a non-Mendelian inheritance [422]. Although no homology was identified with yeast prions, the biochemical analysis performed in this model identified the protein mechanism of infection. Yeast allowed the concept of one-protein as pathogenic element and the idea that a misfolded protein could serve as a template for healthy ones in order to propagate the infection [422, 423]. Moreover, yeast studies demonstrated the molecular mechanisms used by prions to generate different strains, and the mechanism used to overcome species barriers [424, 425].

Mitochondrial dysfunction is another central feature of neurodegeneration. The ability of yeast to ferment helped to identify novel genes involved in the mitochondria function, in condition that can be lethal for other mammalian organism. Yeast permitted also to identify genetic suppressors of ROS aSyn fibrils-mediated production that had failed in presence of A30P or A53T aSyn mutants; suggesting that WT aSyn toxicity mechanisms differ from those of the two familial mutants forms [426].

The finding that key conserved genes within the vesicle mediated transport between the endoplasmic reticulum (ER) and Golgi were conserved between yeast and humans and, most importantly, involved in aSyn pathobiology represented a milestone discovery [71]. Furthermore, co-expression of aSyn and Rab1, the mammalian ortholog of the yeast Ypt1 protein (known yeast gene suppressor of aSyn toxicity), was shown to suppress the loss dopaminergic neurons [71, 73]. Subsequently, other studies identified defects in the vesicular trafficking machinery related with aSyn pathology [427-429]. Yeast deletion mutants were also used to identify genes involved in endocytosis and vacuolar degradation [407].

Finally, yeast present conserved mechanism of cell death and survival, where apoptosis-like process was initially inferred by the similar morphology of dying yeast with mammalian cells. These similarities involved: DNA fragmentation, chromatin condensation, release of cytochrome c, and exposure of phosphatidylserine at the plasma membrane [430]. Furthermore, several apoptotic mammalian regulators present orthologs in the yeast genome; such as apoptosis inducing factor (AIF) and superoxide dismutase (SOD). Although yeast missed one of the key elements of programmed cell death, Bcl2, when the human protein is co-expressed in yeast can protect from mitochondrial cell death. This mechanism demonstrates that heterologous expression of a human protein, Bcl2, absent in yeast, can maintain conserved protein interactions and function in an evolutionary context [431, 432].

Recently, autophagy has become a potential cell death mechanism involved in neurodegeneration. Once again, yeast was used to shed light into possible gene target. The autophagy machinery consists on the highly conserved Atg proteins family, downstream of the target of rapamycin (TOR) kinase. Deletion of Atg proteins in mammalian neurons produced accumulation of misfolded proteins, and genetic and pharmacologic inhibition of TOR resulted neuroprotective [433].

However versatile the yeast model can be, the biological differences between yeast and complex human diseases require validating the insights gained from yeast in mammalian cell models.

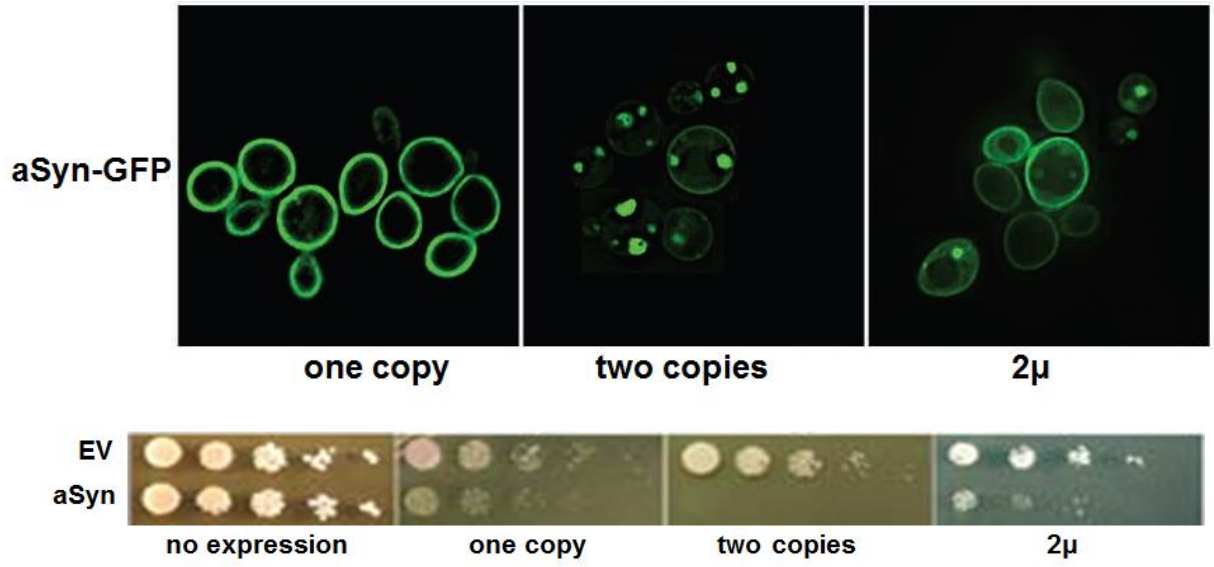


Figure 12. Effects of aSyn expression in yeast cells. On the top panel, fluorescence microscopy of yeast cells expressing aSyn-GFP. In cells carrying one copy of aSyn, the protein localizes mainly at the plasma membrane. Cells with two copies show instead cytoplasmic inclusions (foci), while aSyn cloned into a 2 µ plasmid distributes variably within cells; some presenting foci others membrane-bound pattern. On the bottom panel, growth in solid media of one copy of aSyn (b) has a

minor effect, whereas two copies (c) completely inhibit it. aSyn 2 μ plasmid expression causes mild inhibition of cell growth (d)[434].

1.6.1. *S.cereviasiae* as a model for Parkinson's disease

Two main strategies can be used to model neurodegenerative diseases in yeast [419]. If the gene involved has a known homologous in yeast, it can be over-expressed or deleted and the subsequent phenotype analyzed. For instance, the autosomal-recessive neurodegenerative disorder Friedreich's ataxia is caused by a reduction of a protein called frataxin, encoded by FRDA gene. The yeast FRDA homologue YFH1 has been crucial in determining its function [435]. If the gene involved does not have yeast homologous, the human one is over-expressed in yeast and screened for a phenotype. This situation would particularly match autosomal dominant diseases where the gene product accumulation is one of the major features of the disease, thus the over-expression in yeast can reproduce the main pathological hallmark of the disorder. Classic examples are: Huntington's disease and polyglutamine disorders, Parkinson's disease and synucleinopathies, and Alzheimer's disease [421].

For the purpose of this thesis we will only described the yeast model of PD. aSyn inclusion formation known as LBs are the primary hallmark of a group of disorders called synucleinopathies, including PD. The first yeast model of PD was based on the heterologous expression of different variants of human aSyn. aSyn is conjugated to a green fluorescent protein (GFP), present a galactose inducible promoter and it is cloned within vectors that allow different expression levels. The presence of GFP does not alter the biology of the protein in yeast, thus providing a powerful tool to study aSyn subcellular localization. In this model, at increasing level of expression, aSyn-GFP moves from the plasma membrane to intracytoplasmic inclusions (Fig. 12) [434]. Just doubling aSyn expression levels can lead to the accumulation of aSyn in cytoplasmic inclusions, referred as foci, growth inhibition an ultimately cell death [434]. In addition to aSyn aggregation, using this model, crucial events of PD pathology were also reproduced, namely: defective vesicle trafficking [71, 73, 436], ROS production [437, 438], mitochondrial dysfunction [439], proteasome impairment [434, 437, 440] and lipid-droplet accumulation [407, 441]. Since then, numerous studies were performed using yeast to model different aspects of PD [442-445] and novel mechanism unravelled as autophagy/mitophagy dysfunction [446, 447].

The first yeast genetic screening used a collection of deleted non-essential genes to identify enhancers of aSyn toxicity [448]; the same collection was used to identify modulators of aSyn aggregation and cellular localization, remarkably similar pathways were identified: lipid

metabolism, vesicular transport, and vacuolar degradation [407]. Subsequent microarray analysis at different times point post aSyn expression induction validate these findings, underscoring defects as mitochondrial dysfunction, ER stress, vesicle trafficking and sterol biosynthesis [449]. Screens of small molecules for therapeutic purposes identified two cyclic peptides and four 1,2,3,4-tetrahydroquinolinones as suppressors of aSyn toxicity [449-451]. Other PD related genes have been studied in yeast: for instance, yeast 2-hybrid analysis identified interactors of LRRK2 [452, 453] and another study associated LRRK2 toxicity with defects in endocytic vesicular trafficking and autophagy, observation then validated in primary neuronal models [454]. Yeast models contributed to the functional analysis of the human lysosomal ATPase ATP13A2 [455-458]. The yeast ortholog, YPK9, a vacuolar membrane transporter is a known suppressor of aSyn toxicity [455]; notably, PD associated mutations delocalize Ypk9p, supporting a loss-of-function mechanism [455].

To date, aSyn related toxicity involve several cellular pathways that were initially described in yeast and then corroborate in other animal PD models or successfully recapitulated in yeast.

2. Aims of the study

In this study, we analized the role of aSyn phosphorylation on Ser-129 by Polo-like kinases (PLKs) in established yeast and mammalian cell model of PD, and we addressed the role of tyrosine phosphorylation in an oligodendroglial cell model (OLN) of MSA. The major aim of this project is to understand the potential role of aSyn phosphorylation in terms of cytotoxicity, and oligomerization/aggregation. To this purpose several approaches were used:

1. We co-expressed human kinases previously known to phosphorylate aSyn, namely PLKs, together with aSyn and we assessed aSyn phosphorylation levels on Ser-129, the effect on cell viability and aSyn aggregation. We proceed to validate the results obtained in yeast in mammalian cell models of PD. We co-expressed aSyn and synphilin-1 in cathecolaminergic cell line (CAD) together with the four members of PLKs and we assessed the level of aSyn phosphorylation at Ser-129 and its aggregation state. We then used the same approach in neuroglioma cell line (H4) in order to determine the effect of aSyn Ser-129 phosphorylation on aSyn oligomerization. In this case we used a bimolecular fluorescence complementation (BiFC) assay that enables the direct visualization of protein interactions in their typical subcellular environment.
2. We performed a functional screening in yeast to identify novel kinases involved in aSyn pathobiology. We identified kinases homologous with human ones and co-expressed with human aSyn. We then assessed the effect on aSyn intracellular toxicity, foci formation and phosphorylation levels on Ser-129.
3. We analysed the role of aSyn tyrosine phosphorylation on aSyn cytotoxicity and cellular degeneration in an oligodendroglial cell model (OLN) of MSA (this subproject is carried out as a Neurasync lab secondment in the laboratory of Prof. Poul Henning Jensen, Aarhus University). We co-expressed in oligodendroglia cell line aSyn WT and p25, a tubulin promoting polymerizing protein. The co-expression of p25 and aSyn induces aSyn aggregate dependent toxicity characterized by retraction of the microtubule (MT) cytoskeleton and dependent on Ser-129 phoshorylation. We explored the role of aSyn S129A/Y/F and aSyn Y/F tyrosine phosphorylation mutants (mimics tyrosine non-phosphorylated state) on aSyn toxicity and microtubule retraction.

3. Materials and Methods

Cloning of PLKs, aSyn, and synphilin-1

The yeast expression vectors p426GAL was used to express human aSyn under a galactose-inducible promoter and fused with GFP, as described before [434]. Briefly aSyn was cloned into p426GPD as SpeI-HindIII digested product of PCR amplification. GFP fusion was constructed the correspoding coding sequence as a ClaI-XhoI digested PCR product in frame with aSyn. aSyn fused with GFP was subcloned into p426GAL, p423GAL as SacI-KpnI fragment. Constructs were verified by DNA sequencing.

The PLKs mammalian expression plasmid pCMV6 was a kind gift from Dr. Hilal Lashuel, Ecole Polytechnique Federale de Lausanne, Switzerland. For the PLKs expression in yeast cells, the PLK1 Myc-FLAG gene was excised from pCMV6 and cloned into the ClaI and EcoRI sites of pRS423GAL, while PLK2 Myc-FLAG, PLK3 Myc-FLAG and PLK4 Myc-FLAG genes were excised from pCMV6 and cloned into the ClaI and BamHI sites of p423GAL. The p423GAL PLK2 K111M mutant was generated by site-directed mutagenesis using primers: 5' primer: CAAAGTCTACGCCGCAATGATTATTCCTCACAGCAG and 3' primer: CTGCTGTGAGGAATAATCATTGCGGCGTAGACTTTG (Stratagene: QuikChangeTM Site-Directed Mutagenesis Kit). SynT plasmid (aSynEGFP deletion mutant (WTSynEGFPΔ155)) was previously described [183]; briefly it was generated using PCR with an antisense primer directed to sequences within GFP to remove 465 bp from the C-terminal. aSyn cDNA was then amplified by PCR and cloned into pSI (Promega, Madison, WI, USA) vector. The truncated aSyn-GFP fusion protein has only 93 amino acids of GFP fused to its C-terminal. This fusion protein is no longer fluorescent but has a propensity to aggregate; co-transfection with synphilin-1 further enhances this propensity, thus co-transfection of Syn-T plus synphilin-1 functions as aSyn aggregation model. Synphilin-1 plasmid was also previously described [13, 357].

Cloning of aSynS129A, aSynYF and aSynS129AYF

The oligodendrocytes expression plasmids used were made by Jennifer Skaarup Koffman (former Master student at Dr. Poul Henning Jensen laboratory, Biomedicine Department, Aarhus University). The coding sequences of human aSyn WT and p25 were inserted into XbaI and NotI restriction sites of the pcDNA3.1/zeo(-) vector (Invitrogen). S129A mutation was generated by single nucleotide substitution of TCT to GCT using forward primer 5'-ATAATAGCTAGCATGGATGTATTCATGAAA-3' and reverse primer 5'-ATATATCTTAAGGTCGACCTATGCGGCCCATTCAG-3' (Stratagene: QuikChangeTM Site-Directed Mutagenesis Kit). Triple tyrosine to phenylalanine mutations Y125F, Y133F, and

Y136F were generated by single nucleotide substitution of TAT to TTT (Y125F and Y133F) and TAC to TTC (Y136) using synthesized oligonucleotides as previously described Negro 2002 [371]. The vectors were verified by sequencing at Eurofins MWG Operon using standard CMV primers.

Yeast strains cultures and determination of cell survival

Genotypes of yeast strains and plasmid used are listed in Table 2. Wild-type W303.1a yeast strain cultures were transformed using standard lithium acetate protocol [459]. All strains were grown in Synthetic complete medium without histidine and uracil (SC –His –Ura) for plasmids selection [6.7 g l–1 Yeast Nitrogen Base (BD Biosciences), appropriate amino acid dropout mix (Sunrise Science Products), 1% (w/v) raffinose or 1% (w/v) galactose]. Yeast strains carrying the galactose-inducible aSyn construct were pre-grown in raffinose medium (no repression of the galactose-inducible promoter) prior to galactose medium, to allow synchronous induction of expression.

Yeast cells carrying the galactose-inducible aSyn-GFP construct alone or in combination with PLKs or kinased selected from the ORF library, were pre-grown in SC-His-Ura 1% raffinose for 24 h, then diluted back in order to reach an optical density (OD600) of 0.1 after an overnight growth. For growth assays on solid medium, the cell suspensions were adjusted to OD600nm=0.05±0.005 and used to prepare 1/2 serial dilutions that were applied as spots (4 µl) onto the surface of the solid medium either with SC-His-Ura 1% glucose (control) or SC-His-Ura 1% galactose (induced aSyn and kinases expression) as carbon source. The plates were then incubated at 30°C for at least 48 h.

Table 2. Genotypes of the yeast strain and the plasmids used in this study.

Yeast Strains	Genotype
W303.1a	MATa strain (leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15).

Plasmids	Type of Plasmid
p423GAL-aSyn-GFP	2 µ [434]
p426GAL-aSyn-GFP	2 µ (This study)
p423GAL-PLK1-GFP	2 µ (This study)
p423GAL-PLK2-GFP	2 µ (This study)
p423GAL-PLK2-GFP	2 µ (This study)
p423GAL-PLK4-GFP	2 µ (This study)
pGB1805-KinaseX-HA-FLAG	2 µ (Thermo Fisher Scientific Inc.)

Yeast functional screening

Yeast kinases homologous to human ones as described in literature [460] were analysed using P-POD: Princeton Protein Orthology Database (ppod.princeton.edu). A kinase list based on % of protein identity was genereated using NCBI protein Basic Local Alignment Search Tool (pBLAST; blast.ncbi.nlm.nih.gov). The thirteen kinases chosen for the screening were selected combining protein functional conservation (pBLAST value), brain expression and previous studies published in the PubMed catalog. The corresponding expression vectors were collected from in-house yeast ORF (open reading frame) library of plasmids [461]. Each ORF has been Gateway-cloned into vector pGB1805 in frame with a C-terminal triple affinity tag comprised of 6xHis-HA-C, under control of a galactose-inducible promoter (Thermo Fisher Scientific Inc.,) and –Ura as auxotrophic marker. Vectors, containing the selected kinases, were extracted using a standard miniprep kit (NZYTech, Lda. INOVISAN, Lisbon, Portugal), and then transformed together with the yeast expression vector p423GAL (-His as auxotrophic marker). Human aSyn was expressed under a galactose-inducible promoter and fused with GFP (see section "Cloning of PLKs, aSyn, and synphilin-1") [434]. aSyn and the selected kinases were transformed in the yeast strain W303.1a, using a standard lithium acetate protocol (H. Ito, Y. Fukuda, K. Murata, A. Kimura, J Bacteriol 153, 163, 1983). The cells were then grown and spotted onto SC-His-Ura 1% galactose or SC-His-Ura 1% glucose (control) agar plates (as described in the "Yeast strains cultures and determination of cell survival" section). Modifiers of aSyn toxicity were identified after incubation at 30°C for at least 48 h.

Cell culture and transfections

Mouse CAD cathecolaminergic cells (kind gift from Prof. C. Zurzolo, Instituto Pasteur, Paris) and Human H4 neuroglioma cells (HTB-148; ATCC, Manassas, VA, USA) were maintained in OPTI-MEM (Gibco/Invitrogen corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomicyn, at 37°C under 5 % CO2.

CAD cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and H4 cells with FugeneTM 6 (Roche, Mannheim, Germany) according to the manufacturer's instructions. For immunocytochemistry studies CAD and H4 cell were transfected in 5 cm dish using 0.5 µg of each plasmid DNA per dish. For immunoblotting analysis CAD cells were transfected in 6 well plates using 1 µg of each plasmid DNA per well, while H4 cells were transfected in 10 cm dish using 8 µg of each plasmid DNA per dish. All transfections in CAD and H4 cells were carried out in OPTIMEM + 10 % FCS without penicillin/streptomycin.

Oligodendroglial immortal cell line (OLN WT) is derived from primary Wistar rat brain glial cultures [244]. Cells were cultured at 37°C under 5 % CO2 in Dulbecco's Modified Eagle's

Medium (DMEM) (Lonza) supplemented with 10 % fetal calf serum (FCS), 50 U/ml penicillin, and 50 µg/ml Streptomycin (Gibco BRL). Transient transfection of OLN cell lines for immunoblotting studies was carried out in 6-well plates using FugeneTM 6 (Roche, Mannheim, Germany) according to the manufacturer's instructions, using 0.45 µg of each plasmid DNA per well. For immunocytochemistry studies OLN cells were transfected in 12-well plates using 0.25 µg of each plasmid DNA per well. For MTT studies OLN cells were transfected in 96-well plates using 0.05 µg of each plasmid DNA per well. All transfections in OLN cells were carried out in DMEM + 0,5 % FCS without penicillin/streptomycin.

Compound treatment of transfected H4 cells

H4 cells were stably transfected with GN-link-aSyn+aSyn-GC as previously described (outeiro TF 2008). The PLK2 inhibitor BI2536, APMU (N-[4-(4-aminothieno[2,3-d]pyrimidin-5-yl)phenyl]-N'-(3-methylphenyl)urea), was a kind gift from Prof. Poul Henning Jensen, Institut for Biomedicin – Medicinsk Biokemi, Aarhus Universitet. The inhibitor was diluted in DMSO at a final concentration of 1mM and cells were treated for 2 h, at a final concentration of 1 µM.

Peptide and tyrosine inhibitors treatment of OLN cells

CTSA and CT peptides were designed by Poul Henning Jensen, Institut for Biomedicin – Medicinsk Biokemi, Aarhus Universitet, and produced by Schafer-N, Copenhagen, Denmark. They both correspond to the last 40 aminoacid of aSyn full length protein (100-140). CTSA compared with CT present a substitution in position 129 where an alanine is placed instead of serine (H-MRGSHHHHHHGMARGYGRKKRRPASPGASGKNEEGAPQEGILEDMPVDPDNEAYEMP**A/S**EEGYQDYEPEA-OH); this modification mimics the non-phosphorylated state of aSyn. The peptides present also a TAT domain to permeate cell membranes and a poly-His tag for purification studies. The peptides were dissolved in Dulbecco's Modified Eagle's Medium (DMEM) at a final concentration of 2 mg/ml and used at a working concentration of 5 µM.

For tyrosine inhibitors treatment 20.000 OLN WT cells were plated in each well of a 12 well-plate and 1 cover slip was added on each well. OLN cells were transfected as described in section "Cell culture and transfections". The kinase inhibitor and the vehicle control (DMSO) were added 4 h post-transfection at a final concentration of 5 µM. After 24 h coverslips were processed for immunocytochemistry.

MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) powder was dissolved in RPMI medium to a final concentration of 5 mg/ml and filtrated through 0,2 um filter. 5000

cells were plated per well in a 96 well-plate 24 h prior to transfection. 24 h post transfection (see Cell culture and transfections section) medium culture was removed and cells were gently washed with warm RPMI medium (Sigma Aldrich, St. Louis, MO, USA). 50ul of MTT solution (1:10 dilution of 5 mg/ml stock in RPMI medium) (Sigma Aldrich, St. Louis, MO, USA) was added per well and then incubated for 3 h in the cell incubator at 37 °C. 100 µl of lysis buffer (1% Triton X-100, 0.04 M HCl, diluted in isopropanol) was then added and the plated was incubated for 5 h on a shaking table at RT covered from light. Absorbance was measure at 570 nm using 650 nm as a reference wavelength using VersaMax® microplate spectrophotometer (Molecular Devices, LLC, California, United States).

Western blot analysis

For quantitative analysis equal amount of yeast cells co-expressing aSyn and PLKs were taken 8 h post-induction of aSyn expression, harvested and lysed in Tris-HCl buffer pH 7.4 with glass beads, in the presence of protease and phosphatases inhibitor cocktail (Roche, Mannheim, Germany). Cellular debris was spun down at 2,600 rpm for 5 min and supernatants containing total lysate were collected and sonicated (10 s at 10 mA, Soniprep 150 for Sanyo). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotting was performed following standard procedures.

Mouse CAD cathecolaminergic cells and human H4 neuroglioma cells were lysed with NP-40 buffer 48 h post-transfection (Roche, Mannheim, Germany). Lysates were cleared from debris by a 13,000 rpm centrifugation for 30 s at 4°C and then subjected to sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein resolved on 10% (for PLKs) or 12% (for aSyn) run at 300 V, 30-40 mA. After transfer to nitrocellulose membrane at 300 V, 250 mA, 2 h, the membranes (Bio-Rad) were blocked for 1h at RT in bovine serum albumin BSA 5% in TBS with 0.1% Tween, pH 7.4 (200 mM Tris-HCl pH 7.4, 500 mM NaCl) and then incubated in the same buffer using appropriate antibodies (listed below). Membrane were then washed 3 x 10 min in TBS with 0.1% Tween and incubated with horse radish peroxidase (HRP) labeled secondary antibodies. After washing three times in TBS with 0.1% Tween (TBS-T, pH 7.4), immunoblots were developed using ECL (Millipore, Billerica, MA).

Rat OLN oligodendroglial cells were harvested 24 h post transfection in 30 µl of RIPA buffer (50 mM Tris-Hcl, pH 7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2mM EDTA, 50mM NaF) in the presence of protease and phosphatases inhibitor cocktail (Roche, Mannheim, Germany). Lysates were centrifuged for 3 min at 5,000 rpm to pellet nuclei, subject to SDS-PAGE and protein resolved 4-15 % gradient gels run at 300 V, 30-40 mA. The proteins were electrically transferred to polyvinylidene fluoride membranes (GE Healthcare) at 300 V,

250 mA, 2 h. Blots were blocked for 1 h at RT in BSA 5% in TBS with 0.1% Tween (for phosphorylation studies) or in Milk 5% in TBS with 0.1% Tween, pH 7.4 (200 mM Tris-HCl pH 7.4, 500 mM NaCl); incubated for 1 h at RT or overnight (ON) at 4 °C with appropriate primary antibody. Membranes were then washed 3 x 10 min in TBS with 0.1% Tween-1, and then incubated 1 h at RT in appropriate secondary antibody conjugated to (HRP). The blots were developed by enhanced chemiluminescence (Amersham ECL Western Blotting Detection Reagent, GE Healthcare) using a Fuji LAS-3000 Imager. The primary antibodies used are listed in Table 3.

Immunocytochemistry and microscopy analysis

Fluorescence microscopy of yeast cells was performed with a Zeiss Axiovert 200M Widefield Fluorescence microscope equipped with a digital Axiocam from Zeiss (Objective 100X, Plan-Apochromat, Oil, NA (1.40)). Transformants expressing aSyn alone or with PLK2 or PLK3 were pre-grown on SC-His-Ura 1% raffinose as described above and then on SC-His-Ura 1% galactose medium for 8 h at 30°C with shaking. Yeast cells were subsequently spin down and visualized under the microscope. The proportion of cells presenting aSyn inclusions was then determined by counting at least 1000 cells per strain using ImageJ software. Significance was calculated using Student's two-tailed unpaired t test. A value of $p<0.05$ was considered to be statistically significant.

Transfected CAD cells were fixed with 4% paraformaldehyde in 140 mM NaCl, 10 mM Na-phosphate pH 7.4 (PBS). Cells were permeabilized in PBS containing 0.1% Triton X-100 and blocked in 1.5% normal goat serum containing PBS for 1 h. Cells were incubated with primary antibody for 2 h at RT or overnight at 4°C (mouse anti-aSyn; BD Transduction Laboratories, San Jose, CA, USA; rabbit anti-Venus-synphilin-1; Sigma Aldrich, St. Louis, MO, USA) followed by secondary antibody incubation for 1 h (goat anti-mouse IgG-Alexa488 Invitrogen corporation, Carlsbad, CA, USA and a donkey anti-rabbit IgG-Alexa568, Invitrogen corporation, Carlsbad, CA, USA). Slides were subjected to fluorescence microscopy with a Zeiss Axiovert 200M Widefield Fluorescence microscope equipped with a digital Axiocam from Zeiss (Objective 40X, EC Plan-NeoFluar, Dry, NA (0.75)). The proportion of cells with aSyn inclusions within the population was then determined by counting at least 100 cells per condition using ImageJ software. For BiFC-GFP reconstitution assay, H4 stable cell line expressing aSyn dimers/oligomers was bioimaged as previously described (ADDD)[22]. All data were analyzed using Student's two-tailed unpaired t test. A value of $p<0.05$ was considered to be statistically significant.

OLN cells were cultured on poly-L-lysin coated cover slips for 24 h and fixed in 4 % formaldehyde, PBS for 10 min. Cell membranes were permeabilized by 30 min incubation in

permeabilization buffer (50 mM glycine, 0.1 % Triton X-100, PBS) and were blocked in 3 % BSA, PBS for 30 min. Cells were then incubated in appropriate primary antibodies for 1 h (rabbit anti-aSyn, ASY-1 and rat anti-p25 in-house antibody, PH Jensen laboratory, Biomedicine Department, Aarhus; and mouse anti-tubulin, Sigma Aldrich, St. Louis, MO, USA), washed 3 x 5 min in PBS, incubated 1 h in appropriate fluorescently-labelled secondary antibodies (goat anti-rabbit IgG-Alexa488, goat anti-rat IgG-Alexa568 and goat anti-mouse IgG-Alexa647, Invitrogen corporation, Carlsbad, CA, USA) and washed again. Nuclei were counterstained with DAPI included in the fluorescent mounting medium (DAKO, Denmark). Fluorescent signals were analyzed on a Zeiss Axiovert 200M inverted fluorescence microscope equipped with a camera (Objective 40X, EC Plan-NeoFluar, Dry, NA (0.75)). The microtubule retraction (MT) was quantified as the percentage of αSyn- and p25-positive cells with MT retraction. For each experiment, approximately 100-200 cells were counted for each condition. All data were analyzed using Student's two-tailed unpaired t test. A value of $p<0.05$ was considered to be statistically significant.

Sucrose velocity gradients

Yeast spheroblasts were prepared with the following procedure: yeast cells expressing aSyn alone or together with PLK2 or PLK3 were harvested by centrifugation, washed with sterile water, suspended in spheroplasting solution (Tris pH 7.5 20mM, MgCl2 0.5mM, BME 50 mM, sorbitol 1.2 M and zymolyase 0.5 mg/ml) and incubated at 30ºC for 30 min. Samples were centrifuged at 800 g for 5 min at room temperature and the supernatant was completely removed. Yeast spheroplasts and CAD cells were lysate in a solution containing (Tris pH 7.4 1M, NaCl 5M, SDS 0,4%, Triton X-100 0,2%) with inhibitors for proteases/kinases/phosphatases (Roche, Mannheim, Germany) and were placed 20 min on ice. Then, cells were mechanically disrupted by forcing the solution to pass through a 25G syringe 6 times. 1 mg of total protein was applied on a 5 to 30% sucrose gradient. Centrifugation was conducted at 4°C with a swinging bucket rotor (SW-55Ti rotor, Beckman Instruments, Co., Palo Alto, CA) in a Beckman XL-90 S/N ultracentrifuge at 450,000 rpm for 16 h. Nine fractions were collected, precipitated for 4 h at 4°C in trichloroacetic acid. Proteins were then washed in acetone for three times and resuspended in protein sample buffer (0.5 M Tris-HCl, pH 6.8, Glycerol, 10% (w/v) SDS, 0.1% (w/v) Bromophenol Blue), resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted against total aSyn following standard procedures. The velocity gradient procedure and estimation of the molecular sizes for each fraction were previously described [462].

Table 3. List of primary antibodies used in this study.

Antigen and dilution used	Source	Producer
Alpha-synuclein (aSyn) 1:3000 (yeast studies) 1:1000 (cell studies)	Mouse	BD Transduction Laboratories, San Jose, CA, USA
Alpha-synuclein (aSyn) (ASY-1) 1:1000 (OLN studies)	Rabbit	In-house, PH Jensen laboratory, Aarhus University
phospho-Ser-129 aSyn 1:3000 (yeast and cell studies)	Mouse	Wako Chemicals USA, Inc., Richmond VA, USA
phospho-Tyr-125 aSyn several dilutions tested	Rabbit	Abcam, Cambridge, UK
p25 1:2000 (OLN studies)	Rat	In-house, PH Jensen laboratory, Aarhus University
Venus-synphilin-1 1:2000 (cell studies)	Mouse	Sigma Aldrich, St. Louis, MO, USA
Myc-PLKs 1:2000 (cell and yeast studies)	Mouse	Cell Signaling Technology, Inc., Danvers, MA
GAPDH 1:1000 (yeast studies)	Mouse	Ambion, Cambridgeshire, UK
alpha-tubulin 1:3000 (cell studies)	Mouse	Sigma Aldrich, St. Louis, MO, USA

4. Results

4.1. The role of PLK2 on alpha-synuclein aggregation in yeast

To better understand the function of aSyn phosphorylation, we took advantage of the yeast system to study the molecular effects of Ser-129 phosphorylation on aSyn toxicity and inclusion formation. The *Saccharomyces cerevisiae* yeast strain W303.1a was transformed with human aSyn-GFP cloned into a 2µ vector, under the regulation of a galactose inducible promoter. In these strains, expression of aSyn is moderately toxic to cells, as previously described [434]. Since several studies identified PLKs as one of the most important kinases family involved in aSyn phosphorylation [331], the same strain was then transformed with the four members of human PLKs. PLKs-myc were also cloned in a 2µ vector, under galactose promoter.

We determined the levels of Ser-129 phosphorylation by immunoblotting. As previously described, co-expression of aSyn with PLK2 and PLK3 resulted in a pronounced increase in the levels of aSyn Ser-129 phosphorylation, while PLK4 had no significant effect (Fig. 13A). PLK1 blot band was much lower compared to the other kinases (Fig. 13A), probably either due to its low protein expression levels or higher insolubility, precluding further studies on the effect of this kinase on aSyn phosphorylation; therefore, we focused on PLK2 and PLK3.

Initially, we investigated whether PLK2 or PLK3 altered the cytotoxicity induced by aSyn in our yeast model. Using spotting assay, we found that co-expression of PLK2 with aSyn reduced cell growth, while PLK3 had no effect (Fig. 13B).

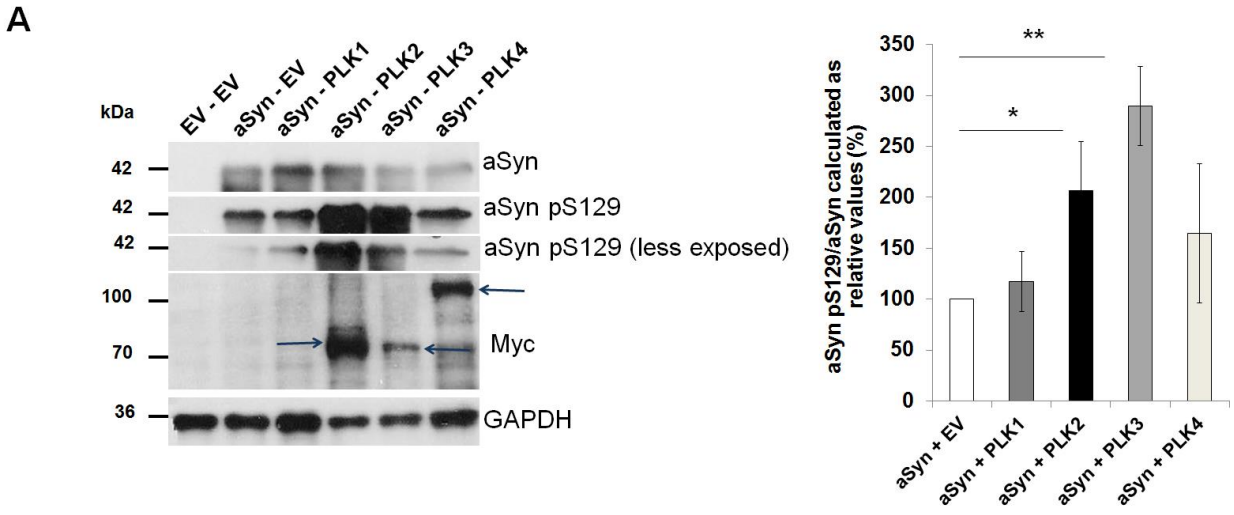
Next, we investigated whether PLK2-induced toxicity was associated with the formation of intracellular inclusions. Interestingly, while 50% of the cells expressing aSyn alone formed fluorescent foci (Fig. 13C), co-expression of PLK2 resulted in a 10% increase of foci-positive cells (Fig. 13C). Remarkably, PLK3 although able to increase aSyn phosphorylation on Ser-129, did not affect either the percentage of cells bearing aSyn foci or cell growth.

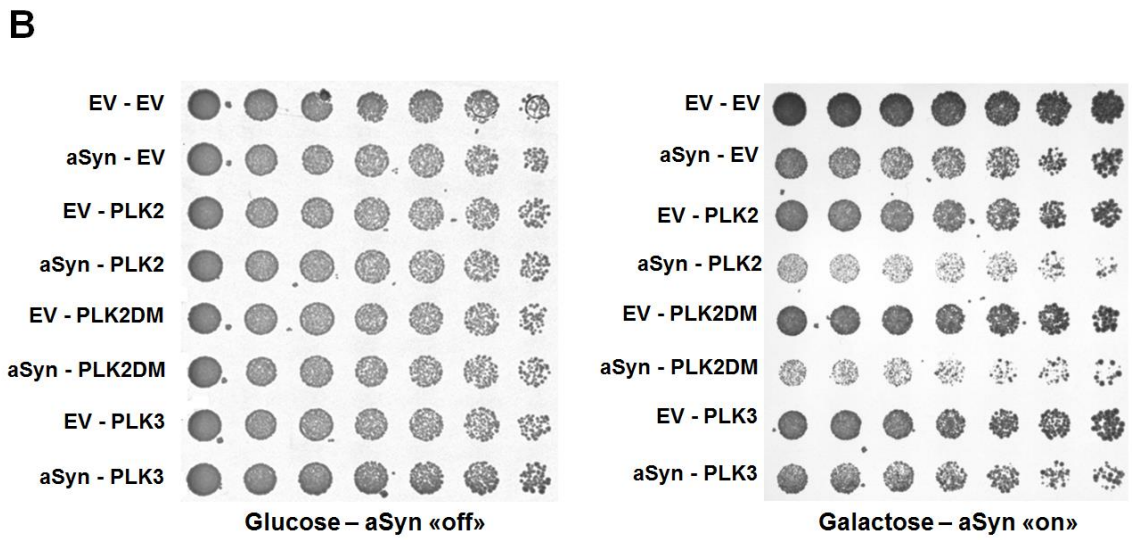
Thus, to establish the role of PLK2 mediated phosphorylation on aSyn toxicity and inclusion formation, experiments were carried out in the presence of a kinase dead mutant (DM) of PLK2, carrying a K111M substitution [463]. As expected, the PLK2 kinase dead mutant (PLK2DM) prevented the phosphorylation on Ser-129 without affecting the levels of aSyn (Fig. 13D). We then tested the effect of PLK2DM on the cytotoxicity of aSyn by spotting assays, and we detected a decrease in cell growth, as observed for the WT PLK2 protein. We next asked whether PLK2DM co-expression affected the distribution of aSyn in yeast cells. Surprisingly, we did not observe any difference in the percentage of cells with aSyn foci compared with the one co-expressing WT PLK2 (Fig. 13C). We then analyzed aSyn co-expression with PLK4, a member of the PLK family that was unable to increase aSyn phosphorylation levels on Ser-129

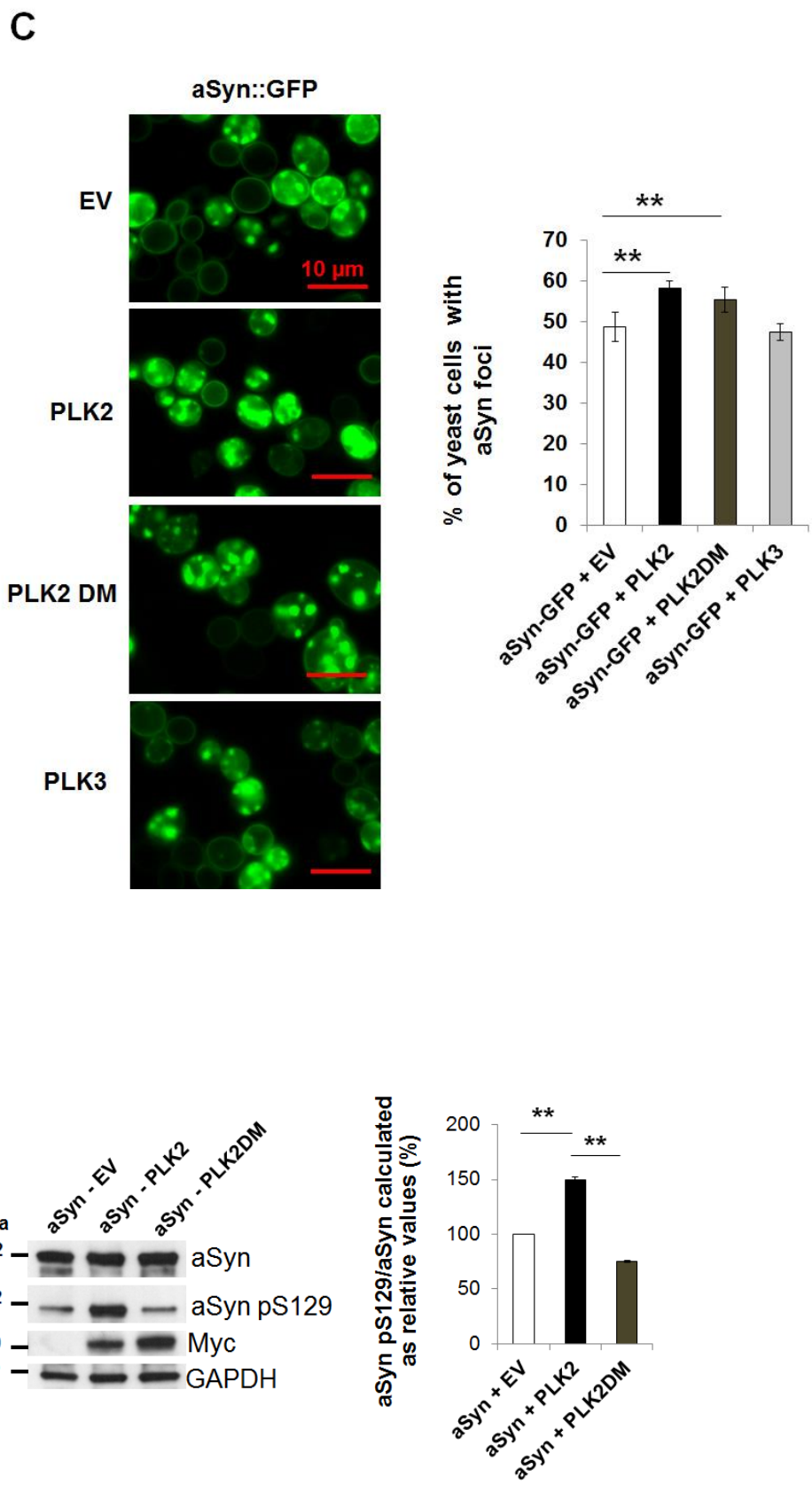
(Fig. 13A). We found that PLK4 co-expression did not alter aSyn foci formation and toxicity (Fig 13F).

Altogether these results demonstrate that the phenotype observed is not only correlated with aSyn phosphorylation on Ser-129 but it is specific for PLK2; suggesting that the role of PLK2 on aSyn inclusion formation and toxicity might result from additional effects that are not solely dependent on PLK2 kinase activity and, consequently, on the direct phosphorylation of aSyn on Ser-129.

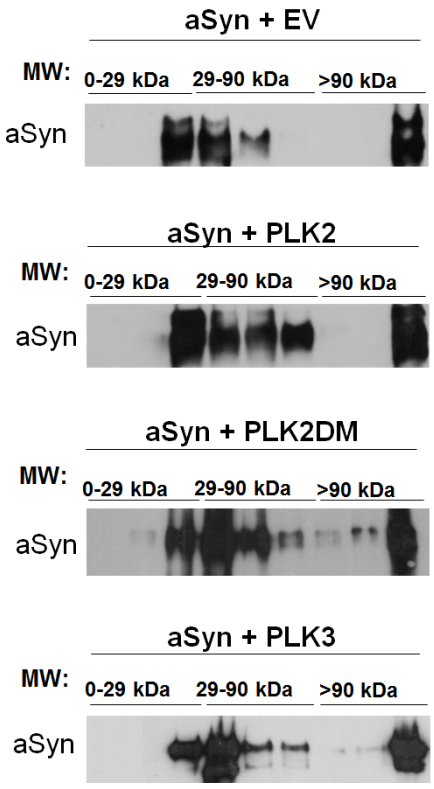
Next, in order to assess the biochemical state of aSyn-GFP in the fluorescent foci we performed ultracentrifugation analysis in sucrose gradients. We observed an increase in the levels of aSyn species with higher molecular weight in cells co-expressing PLK2, PLK2DM and PLK3 (Fig. 13E). PLK2DM promoted the accumulation of similar types of aSyn-GFP species to those formed in the presence of PLK2 (Fig. 13E), confirming that the effect of PLK2 on aSyn inclusion formation is independent from its ability to phosphorylate aSyn on Ser-129.






C
aSyn::GFP
EV
PLK2
PLK2 DM
PLK3
10 µm
% of yeast cells with aSyn foci
**
**
70
60
50
40
30
20
10
0
aSyn-GFP + EV
aSyn-GFP + PLK2
aSyn-GFP + PLK2DM
aSyn-GFP + PLK3
D
aSyn - EV
aSyn - PLK2
aSyn - PLK2DM
kDa
42
aSyn
42
aSyn pS129
70
Myc
36
GAPDH
aSyn pS129/aSyn calculated as relative values (%)
**
**
200
150
100
50
0
aSyn + EV
aSyn + PLK2
aSyn + PLK2DM

E


aSyn + EV
MW:
0-29 kDa
29-90 kDa
>90 kDa
aSyn
aSyn + PLK2
MW:
0-29 kDa
29-90 kDa
>90 kDa
aSyn
aSyn + PLK2DM
MW:
0-29 kDa
29-90 kDa
>90 kDa
aSyn
aSyn + PLK3
MW:
0-29 kDa
29-90 kDa
>90 kDa
aSyn

F

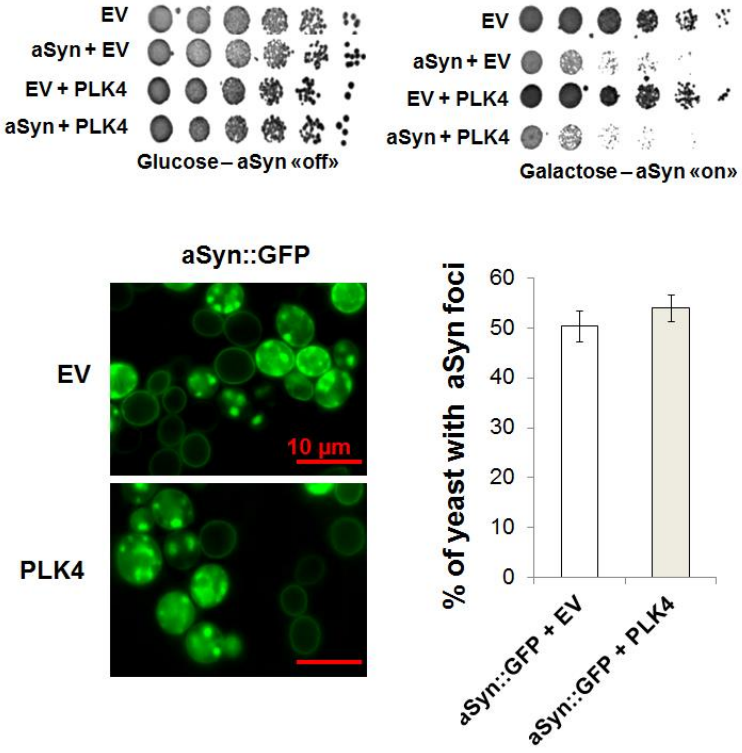
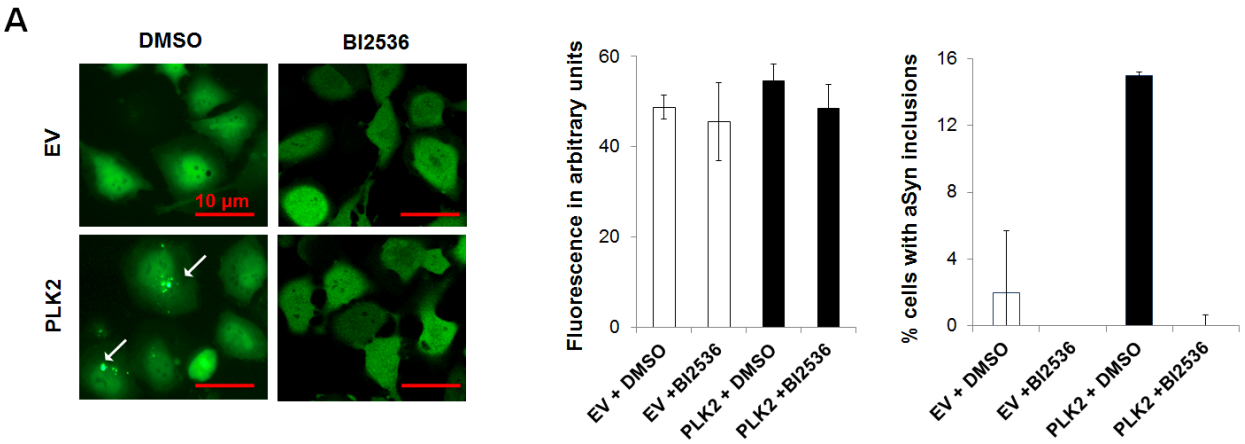

EV
aSyn + EV
EV + PLK4
aSyn + PLK4
Glucose – aSyn «off»
EV
aSyn + EV
EV + PLK4
aSyn + PLK4
Galactose – aSyn «on»
aSyn::GFP
EV
PLK4
10 µm
% of yeast with aSyn foci
60
50
40
30
20
10
0
aSyn::GFP + EV
aSyn::GFP + PLK4

Figure 13. PLK2 increases aSyn Ser-129 phosphorylation, cytotoxicity and foci formation in yeast. A. Phosphorylation levels of aSyn on Ser-129 (pSER129) when expressed either alone (aSyn + empty vector (EV)) or with PLK1 (68 kDa), PLK2 (78 kDa), PLK3 (71 kDa) and PLK4 (104 kDa) determined by immunoblotting (left panel). pSER129 overexposed blot showing phosphorylation levels in all the conditions examined. Densitometric analysis of the immunodetection of aSyn Ser-129 phosphorylation levels were normalized for the total amount of aSyn (mean ± s.e.m.) and relative to the aSyn + EV condition (upper panel). **B.** Spotting assay of yeast cultures co-expressing aSyn (aSyn + EV) or empty vector (EV) with PLK2 or PLK3 or PLK2DM (dead kinase mutant version of PLK2). **C.** Fluorescence microscopy and quantification of the number of cells presenting aSyn foci in cells expressing aSyn-GFP fusion protein alone (aSyn-GFP + EV) or together with PLK2, PLK3 or PLK2DM. **D.** Phosphorylation levels of aSyn on Ser-129 when expressed either alone (aSyn + EV) or together with either PLK2 or PLK2DM determined by immunoblotting (upper panel). Densitometric analysis of the Ser-129 phosphorylation levels normalized for the total amount of aSyn (mean ± s.e.m.) (upper right panel). **E.** The oligomeric species formed in yeast cells expressing aSyn alone (aSyn + EV) or with PLK2 or PLK2DM or PLK3 were resolved using sucrose gradients. The resulting fractions were applied to a SDS-page gel followed by immunoblotting with an antibody anti-aSyn (lower panel). **F.** Spotting assay of yeast cultures co-expressing aSyn (aSyn + EV) or empty vector (EV) with PLK4. Fluorescence microscopy and quantification of the number of cells presenting aSyn foci in cells expressing: aSyn-GFP alone (aSyn-GFP + EV) or together with PLK4. All the data shown are representative of at least three independent experiments. Statistical analysis was performed using two-tailed Student's *t* test for unpaired data (*=$p<0.05$, **=$p<0.005$).

4.2. The role of PLK2 on alpha-synuclein aggregation in mammalian cell models of PD

In order to validate the results obtained in yeast, we investigated the effect of PLK2 on aSyn oligomerization in a neuroglioma human cell line (H4 cells). First, we assessed the effect of PLK2 on aSyn oligomerization using bimolecular fluorescence complementation assay (BiFC) (Fig. 14A) [186]. Cells stably expressing the aSyn BiFC constructs were transfected with PLK2 and analyzed by fluorescence microscopy (Fig. 14A). The overall fluorescence of the cells was not changed in the presence of PLK2. However, in 10% of the cells co-expressing PLK2 we observed the presence of punctate foci, suggesting oligomers were recruited into larger inclusions (Fig. 14A). We then investigated the role of PLK2 on inclusion formation using the BI2536 PLK2 inhibitor [330]. This treatment eliminated aSyn inclusions (Fig. 14B). In parallel, we evaluated aSyn expression levels as well as Ser-129 phosphorylation levels by immunoblotting analysis. As expected, we detected a significant increase in aSyn Ser-129 phosphorylation in the presence of PLK2, while treatment with BI2536 reduced phosphorylation (Fig. 14B), indicating that the BI2536 PLK2 inhibitor was effective.



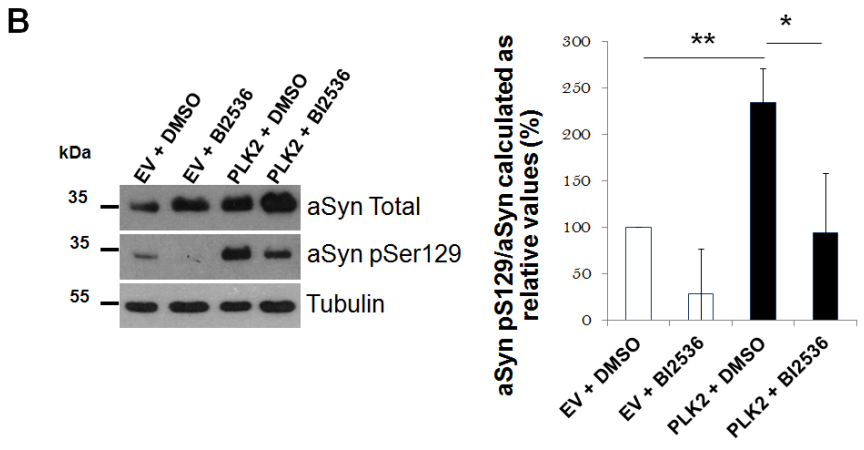


Figure 14. PLK2 does not interfere with aSyn oligomerization. A. Microscopy analysis of H4 cells stably transfected with GN-link-aSyn+aSyn-GC and transiently co-transfected either with PLK2 or an empty vector (EV), in the presence (BI2536) or absence (DMSO) of a kinase inhibitor. aSyn fluorescence intensity is quantified in arbitrary units. The percentage of cells with aSyn inclusions is shown. **B.** H4 cells stably transfected with GN-link-aSyn+aSyn-GC were immunoblotted 48 h post transient co-transfection either with PLK2 or empty control (EV), in the presence (BI2536) or absence (DMSO) of a kinase inhibitor, using antibodies against aSyn phosphorylated on Ser-129 and total aSyn. Ser-129 phosphorylation levels were normalized for the total amount of aSyn (mean ± s.e.m.) and relative to the EV + DMSO condition. All data presented are representative of three independent experiments. Statistical analysis was performed using two-tailed Student's *t* test for unpaired data (*=p<0.05), (**=p<0.005).

To further confirm the effect of PLK2 on aSyn aggregation propensity, we used another mammalian cell system. PLK2 was co-expressed with aSyn and synphilin-1, an established paradigm of aSyn aggregation which results in the formation of LB-like inclusions [183]. Synphilin-1 is a known aSyn interacting protein [13], present in the core of LBs [464]. A cathecolaminergic mouse cell line (CAD) was co-transfected with aSyn and synphilin-1 together with each of the four PLKs. We then evaluated the levels of aSyn and Ser-129 phosphorylation by immunoblotting analysis. Both PLK2 and PLK3 induced aSyn Ser-129 phosphorylation while PLK1 had no effect (Fig. 15A). The lower expression levels of PLK4 precluded further conclusions on the effects of this kinase (Fig. 15A).

Interestingly, co-expression of aSyn with PLK3, but not PLK2, resulted in a significant reduction in the levels of aSyn (Fig. 15A), in agreement with previous studies [334]. Thus, it was not possible to further evaluate the role of PLK3 on aSyn phosphorylation or aggregation using this cell model.

Next, we investigated the effect of PLK2 on aSyn aggregation by immunocytochemistry. We found that PLK2 slightly altered the percentage of cells displaying aSyn inclusions, although

this did not reach statistical significance (Fig. 15B). Nevertheless, the accumulation of larger aSyn inclusions was observed in the presence of PLK2, confirming an effect on aSyn aggregation propensity (Fig. 15B).

To assess the biochemical nature of the aSyn aggregates formed in the presence of PLK2, we performed ultracentrifugation in sucrose gradient. In agreement with the data obtained in yeast, we observed an increase of aSyn higher order oligomeric species (Fig. 15C).

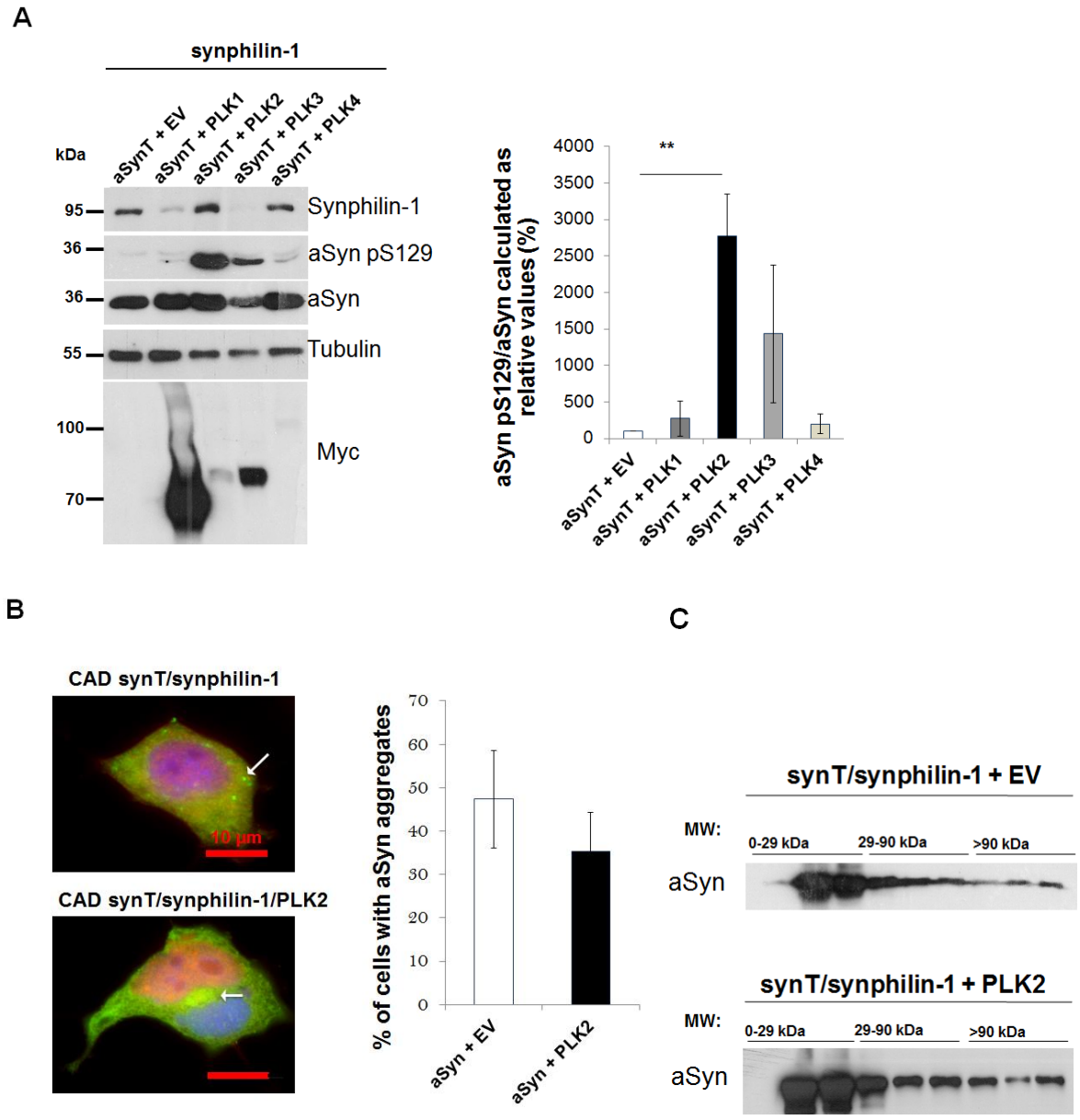


Figure 15. PLK2 induces aSyn phosphorylation and increases the size of aSyn aggregates in CAD cells. A. CAD cells were transiently transfected with aSynEGFPΔ155 (aSynT + EV), synphilin-1 and PLK1, PLK2, PLK3 and PLK4. Cells were lysed and immunoblotted for phosphorylated aSyn, total aSyn, synphilin-1 and PLKs (myc-tag). Ser-129 phosphorylation levels were normalized for the total amount of aSyn (mean ± s.e.m.) and relative to the aSynT + EV. **B.** aSynT (aSynEGFPΔ155), synphilin-1 and PLK2 expressing cells were immunostained for aSyn and synphilin-1. The number of cells containing aSyn aggregates is plotted as a percentage of total transfected cells and relative to

the aSynT + EV condition. **C.** Lysates of CAD cells expressing aSynT (aSynEGFPΔ155) alone or with PLK2 were ultracentrifuged in sucrose gradient and analyzed by immunoblotting using antibodies against aSyn phosphorylated on Ser-129 and total aSyn. All data presented are representative of three independent experiments. Statistical analysis was performed using two-tailed Student's *t* test for unpaired data (**=p<0.005).

The results described in the section 4.2 are part of the following pubblication:

Basso E., Antas P., Marijanovic Z., Gonçalves S., Tenreiro S. and Outeiro TF. "PLK2 modulates a-synuclein aggregation in yeast and mammalian cells". Mol Neurobiol. 2013 May 17.

4.3. Yeast functional screening to identify novel kinases involved in alpha-synuclein pathobiology

The yeast kinome [460] was analyzed using the P-POD: Princeton Protein Orthology Database (as described in materials and methods section). This is a database system that permits to find the phylogenetic relationships among orthologs to a query gene, and visualize them in an evolutionary context. The database displays also results collected from the literature linking to relevant human disease and gene information via the OMIM, model organisms, and sequence databases.

17 kinases were selected according to three features: functional conservation with human orthologous, expression in the nervous system and previous data available in the PubMed catalogue (Table 4). Among the selected kinases *ATG1*, *KNS1* and *NRK1* were included since they were found differently expressed in yeast strains expressing aSyn WT compared to aSyn S129A phosphorylation mutant (microarray analysis performed by Sandra Tenreiro in our laboratory): *ATG1* and *KNS1* were up-regulated in the S129A mutant compared to the WT, while *NRK1* was down-regulated. After retrieving the corresponding plasmids from our ORF library [461], the yeast strain W303.1a was transformed with aSynWT-GFP, cloned into a 2µ vector under the regulation of a galactose inducible promoter, and each of the 17 kinases or the corresponding empty vector. The kinases-HA tagged were also cloned in a 2µ vector, under galactose promoter.

The screen to assess the kinases effect consisted of three main assays:

1. Growth assay: using spotting assay (a previously described phenotypic assay).
2. Immunoblotting analysis: protein expression and aSyn phosphorylation on Ser-129 was evaluated by SDS-PAGE.
3. aSyn-GFP foci: the number of cells presenting aSyn-GFP positive inclusions was quantified by microscopy 8 hours post induction of expression.

Table 4. Yeast kinases selected for the functional screening.

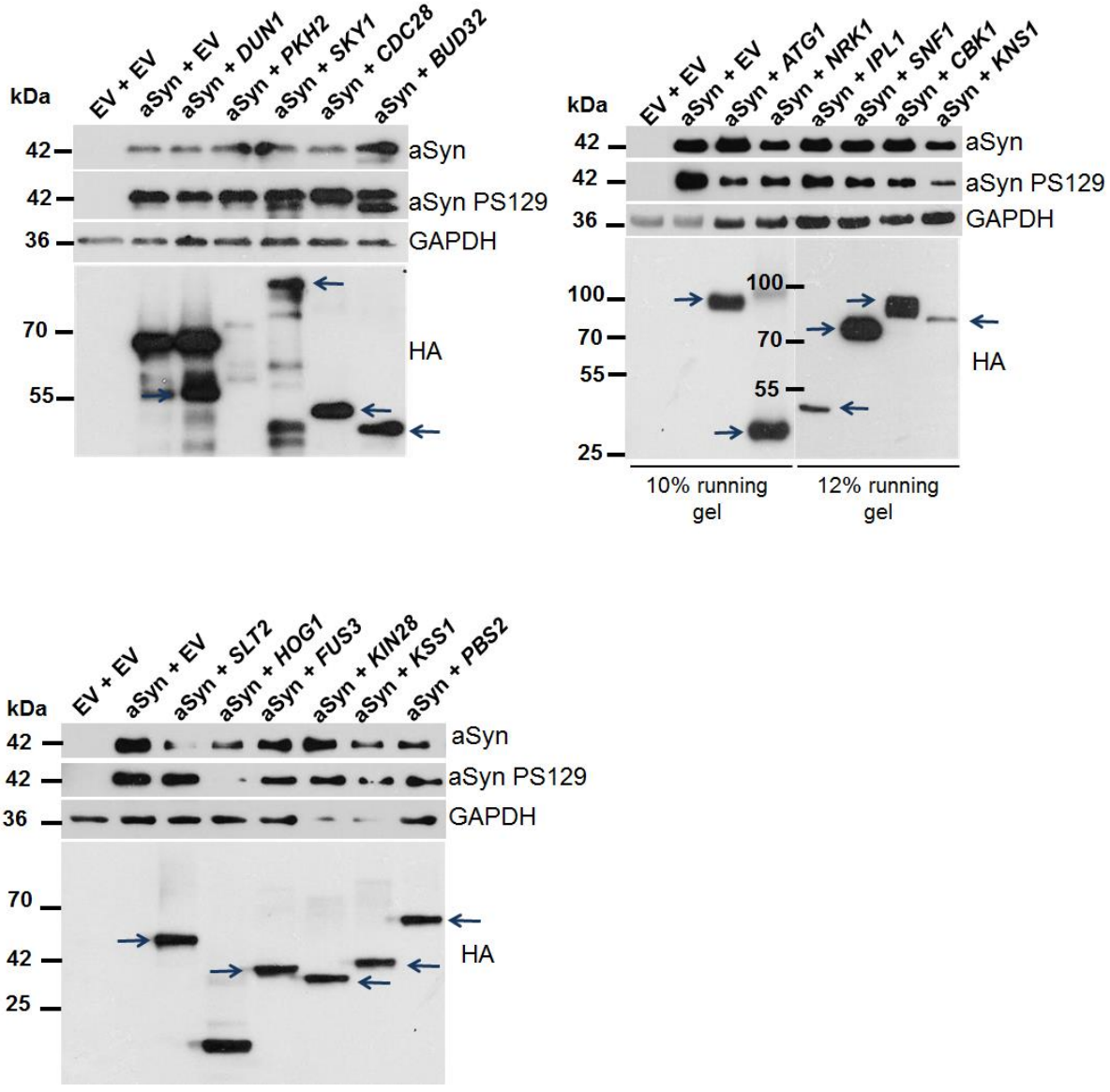
Yeast Kinases	Protein Identity (pBLAST)	Human orthologous	Expression	Reference
DUN1 **CAMK family**	37%	Serine/threonine-protein kinase CHK2	Ubiquitous	[465]
PKH2 **PKA related family**	46%	3-phosphoinositide-dependent protein kinase 1 (PDK1)	Ubiquitous	[466, 467]
SKY1 **CLK family**	35%	Serine/threonine-protein kinase SRPK1	Highly expressed in brain	[468]
CDC28 **CDK family**	98%	Cyclin-dependent kinase 2	Ubiquitous	[469]
BUD32	99%	TP53 regulating kinase (TP53RK)	Ubiquitous	[470]
NRK1 **PAK/Ste20 family**	54%	Serine/threonine-protein kinase 24	Isoform A: Ubiquitous Isoform B: Brain	[471]
ATG1 **ATG family**	34%	Serine/threonine-protein kinase ULK1	Ubiquitous	[472]
IPL1 **AUR family**	44%	Serine/threonine-protein kinase aurora-A	Found at the neuritis hillock in developing neuron.	[473]
SNF1 **AMPK family**	41%	5'-AMP-activated protein kinase catalytic subunit alpha-1 and alpha-2	Ubiquitous	[474]
CBK1 **nuclear Dbf2-related (NDR) family**	48%	Serine/threonine-protein kinase 38-like	Ubiquitous	[475]
KNS1 **CLK family**	40%	Dual specificity protein kinase CLK4	Expressed in liver, kidney, heart, muscle, brain and endothelial cells	[476]
SLT2 **MAPK family**	43%	Mitogen-activated protein kinase 7	Adult tissue	[477]
HOG1 **MAPK family**	52%	Mitogen-activated protein kinase 2	Brain, heart, placenta, pancreas and skeletal muscle. Expressed to a lesser extent in lung, liver and kidney	[478]
FUS3 **MAPK family**	50%	Mitogen-activated protein kinase 3	Ubiquitous	[479]
KIN28 **CDK family**	48%	Cell division protein kinase 7	Ubiquitous	[480]
KSS1 **MAPK family**	50%	Mitogen-activated protein kinase 1	Ubiquitous	[481]
PBS2 **STE7 family**	42%	Dual specificity mitogen-activated protein kinase kinase 1	Ubiquitous	[482]

After performing the spotting assays,, two kinases consistently showed a significant increase in growth impairment compared with the condition in which solely aSyn was expressed: Atg1 and Sky1 (Fig. 16B). Atg1 is a Ser/Thr kinase required for the cytoplasm to vacuole targeting pathway (Ctv), whose over-expression is used as a model to induce autophagy both in yeast and mammalian cell lines [483]. Sky1 is a Ser/Thr kinase regulating proteins involved in mRNA

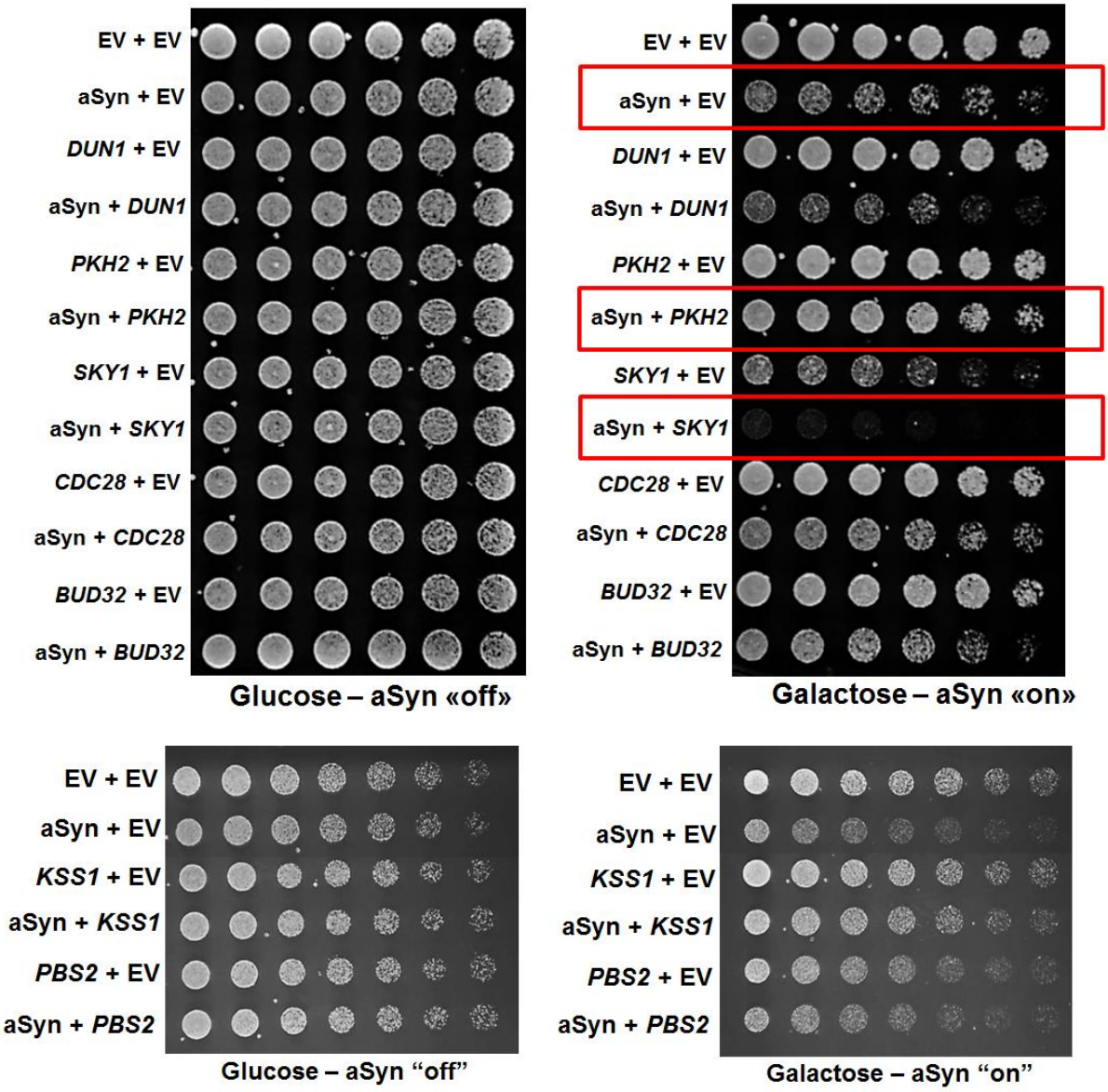
metabolism [484]. Conversely, only one kinase reverted the phenotype, Pkh2, augmenting yeast cell growth in aSyn dependent manner (Fig. 16B). Pkh2 is a Ser/Thr kinase involved in sphingolipid-mediated signaling pathway that controls endocytosis and activates signaling cascade components required for maintenance of cell wall integrity [485], whereas the human homologous is involved in insulin signaling pathway [466].

We next performed immunoblotting analysis to characterize aSyn phosphorylation levels on Ser-129. Data demonstrated that none of the 17 kinases seem to significantly affect aSyn phosphorylation (Fig. 16A,C). Furthermore expression of Hog1 and Pkh2 was not detected, thus the phenotype observed with Pkh2 might not be associated with the kinase in study. Nonetheless, for the other kinases studied, further experimental data are necessary to evaluate phosphorylation levels of other aSyn known residues, such as Ser-87 or Tyr-125.

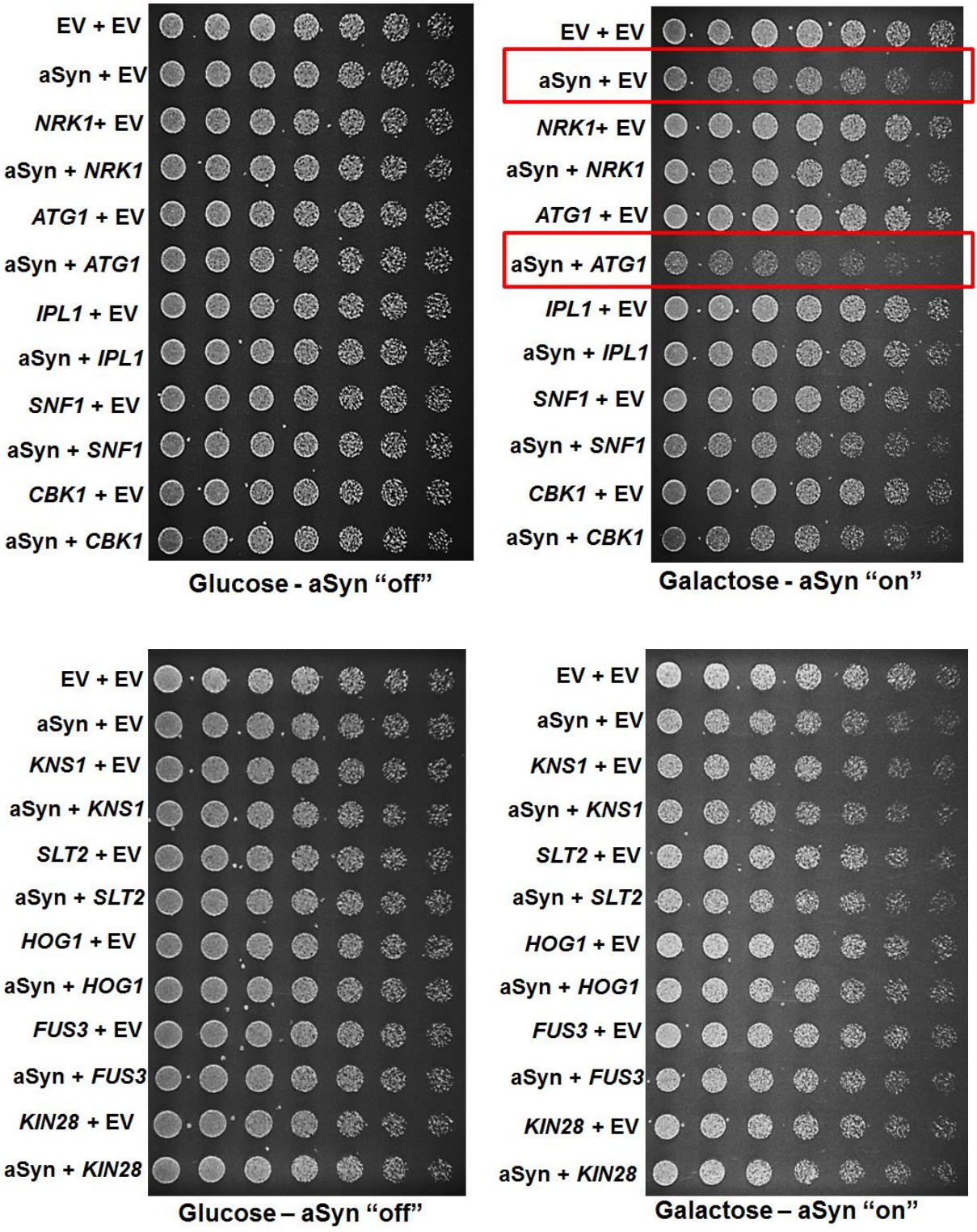
A



B


EV + EV
aSyn + EV
DUN1 + EV
aSyn + DUN1
PKH2 + EV
aSyn + PKH2
SKY1 + EV
aSyn + SKY1
CDC28 + EV
aSyn + CDC28
BUD32 + EV
aSyn + BUD32
Glucose – aSyn «off»
Galactose – aSyn «on»
EV + EV
aSyn + EV
KSS1 + EV
aSyn + KSS1
PBS2 + EV
aSyn + PBS2
Glucose – aSyn "off"
Galactose – aSyn "on"

B


EV + EV
aSyn + EV
NRK1+ EV
aSyn + NRK1
ATG1 + EV
aSyn + ATG1
IPL1 + EV
aSyn + IPL1
SNF1 + EV
aSyn + SNF1
CBK1 + EV
aSyn + CBK1
Glucose - aSyn "off"
Galactose - aSyn "on"
EV + EV
aSyn + EV
KNS1 + EV
aSyn + KNS1
SLT2 + EV
aSyn + SLT2
HOG1 + EV
aSyn + HOG1
FUS3 + EV
aSyn + FUS3
KIN28 + EV
aSyn + KIN28
Glucose – aSyn "off"
Galactose – aSyn "on"

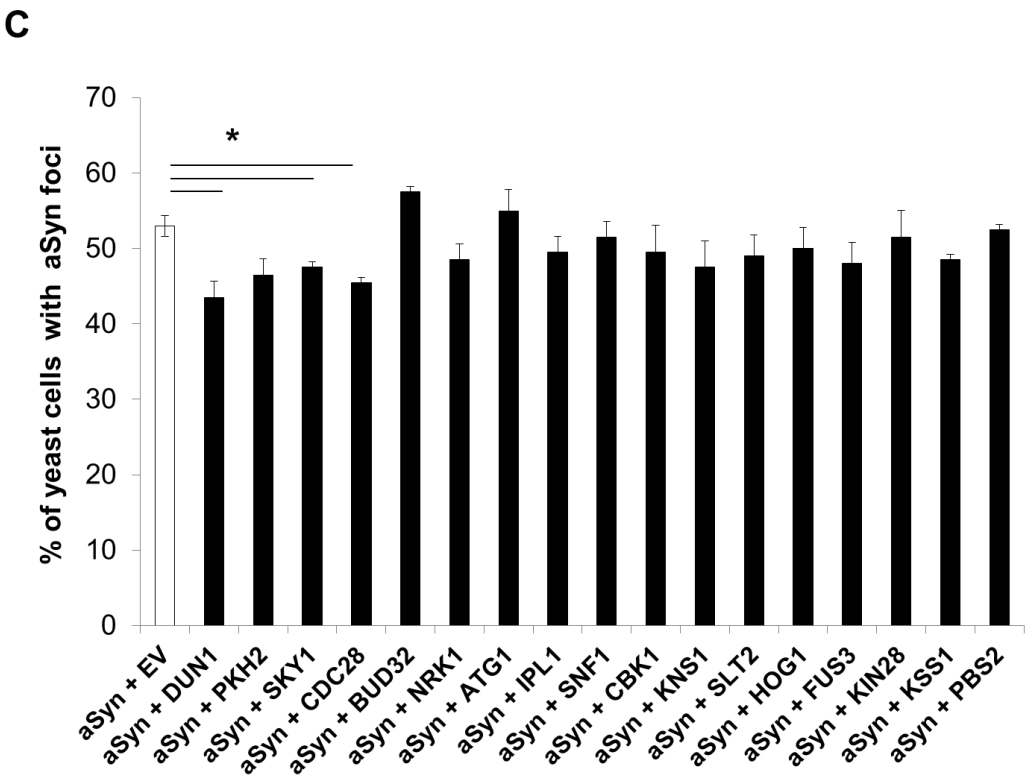
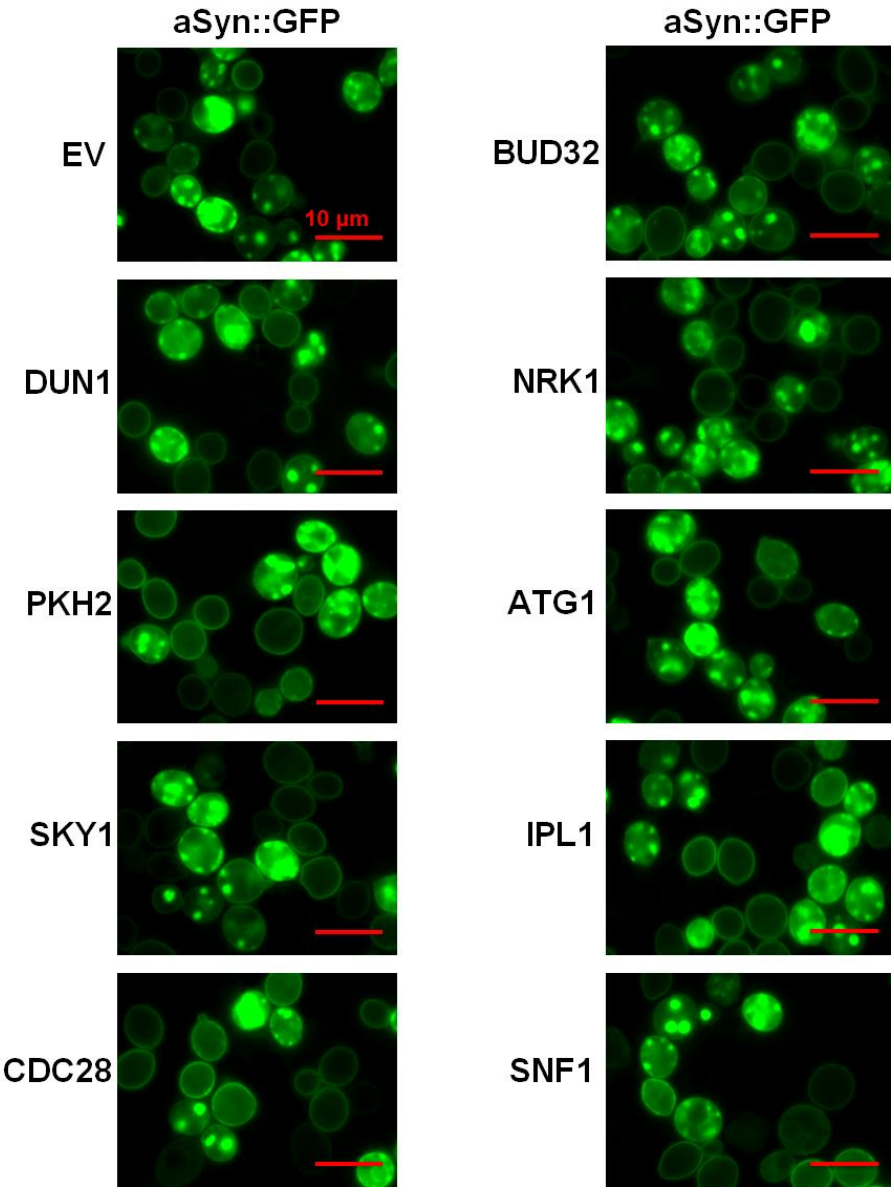


Figure 16. Atg1 and Sky1 decreases aSyn yeast dependent cell growth, while PKH2 increases it A. Immunoblotting analysis of total and aSyn phosphorylated on Ser-129 co-expressed with empty vector (aSyn + EV) or with the indicated kinases after 8 hours of expression induction. Kinases expression was detected using an antibody against HA flag. Correspondent MW is indicated by arrows, where absent, the specific kinase was not detected or the MW was not the predicted one. **B.** Spotting assay of yeast cultures co-expressing aSyn (aSyn + EV) or empty vector (EV) with 17 different kinases selected for the screening. **C.** Densitometric analysis of the immunodetection of aSyn Ser-129 phosphorylation levels were normalized for the total amount of aSyn (mean ± s.e.m.) and relative to the aSyn + EV condition. All the data shown are representative of two independent experiments.

Next, we investigated whether any of the kinases affected the formation of aSyn foci. It has been previously established that 50% of the cells 8 hours post induction of aSyn expression (cloned in a 2µ vector) form fluorescent foci (Fig. 17). Outstandingly, none of the kinases analyzed increased significantly the number of cells bearing aSyn foci (Fig. 17), while Dun1, Sky1 and Cdc28 decreased it significantly. Sky1 expression in particular, decreases both yeast growth and foci formation; anyway further experimental data will be necessary to establish a potential correlation between the two phenotypes observed. Additional data will aim to characterize which types of aSyn species are being formed or will evaluate possible differences in the number of foci per cell or their size.

Altogether these data produced useful information to further investigate two new kinases, Atg1 and Sky1, involved in aSyn pathobiology.



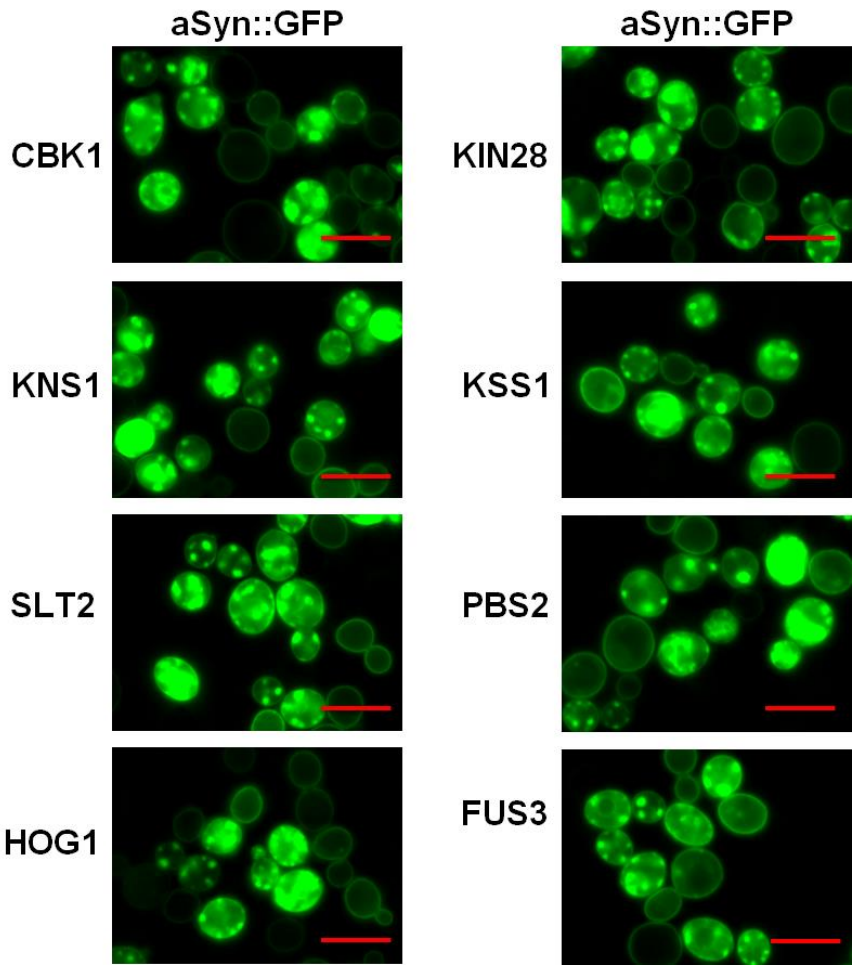


Figure 17. Cells bearing aSyn foci upon co-expression with the selected kinases. Representative fluorescence images of cells expressing aSyn alone (aSyn-GPF + EV) or with the selected kinases (top panel). On the bottom, percentage of cells presenting aSyn foci in cells co-expressing aSyn-GFP fusion with empty vector (aSyn-GFP + EV) or with the indicated kinases. All the data shown are representative of two independent experiments. Statistical analysis was performed using two-tailed Student's *t* test for unpaired data (*=p<0.05).

4.4. The role of alpha-synuclein phosphorylation on microtubule retraction in oligodendroglial cells

To model the molecular events underlying MSA, we used a cellular model based on the co-expression of aSyn and p25 in the rat OLN WT oligodendrocyte cell line [246]. Co-expression of these two proteins promotes microtubule dysfunction within 24 h and rounding up of the cell that ultimately leads to activation of apoptotic processes. This result represents a combined effect aSyn-specific and p25 because expression of either aSyn or p25 alone does not induce any disruption of the MT network. The authors then addressed the role of Ser-129 phosphorylation using a mutant that mimics the non-phosphorylated S129A state of the protein (SA). The SA mutant prevented MT retraction and cell degeneration [246]. Furthermore, the role of a known aSyn aggregation inhibitor, ASI-1D [486] was assessed. ASI-1D was identified based on a library compound screening and comprises mainly the NAC region of aSyn; treatment with ASI-1D reduced MT retraction, indicating that aSyn aggregation is involved in the observed phenotype [246].

To assess the role of tyrosine phosphorylation in the above oligodendroglial cellular model, we constructed two different phosphorylation mutants: aSynSAYF and aSynYF. In these constructs three tyrosine residues in position 125, 133 and 136 were mutated to a phenylalanine, a substitution that by removing a hydroxyl group mimics the non-phosphorylated state of the protein. We evaluated the effect on MT retraction 24 h post transfection by immunostaining (Fig. 18A). Our data demonstrated that the absence of phosphorylated tyrosine residues disrupted the protection induced by the aSynSA mutant (Fig. 18B), suggesting an important role for tyrosine phosphorylation in this model. Moreover, the percentage of cells displaying MT retraction reached the same value of aSyn WT condition, further corroborating the importance of available aSyn tyrosine residues in the microtubule dynamics and in the cellular architecture. We then performed immunoblotting analysis to determine protein expression levels (Fig. 18C). We noticed that aSynYF expression levels were lower compared to the others proteins; we hipothezied that this mutant may form higher insoluble aSyn species. Conclusively, tyrosine residues in the C-terminal of aSyn are involved in the degenerative phenotype in the oligodendroglial model co-expressing aSyn and p25.

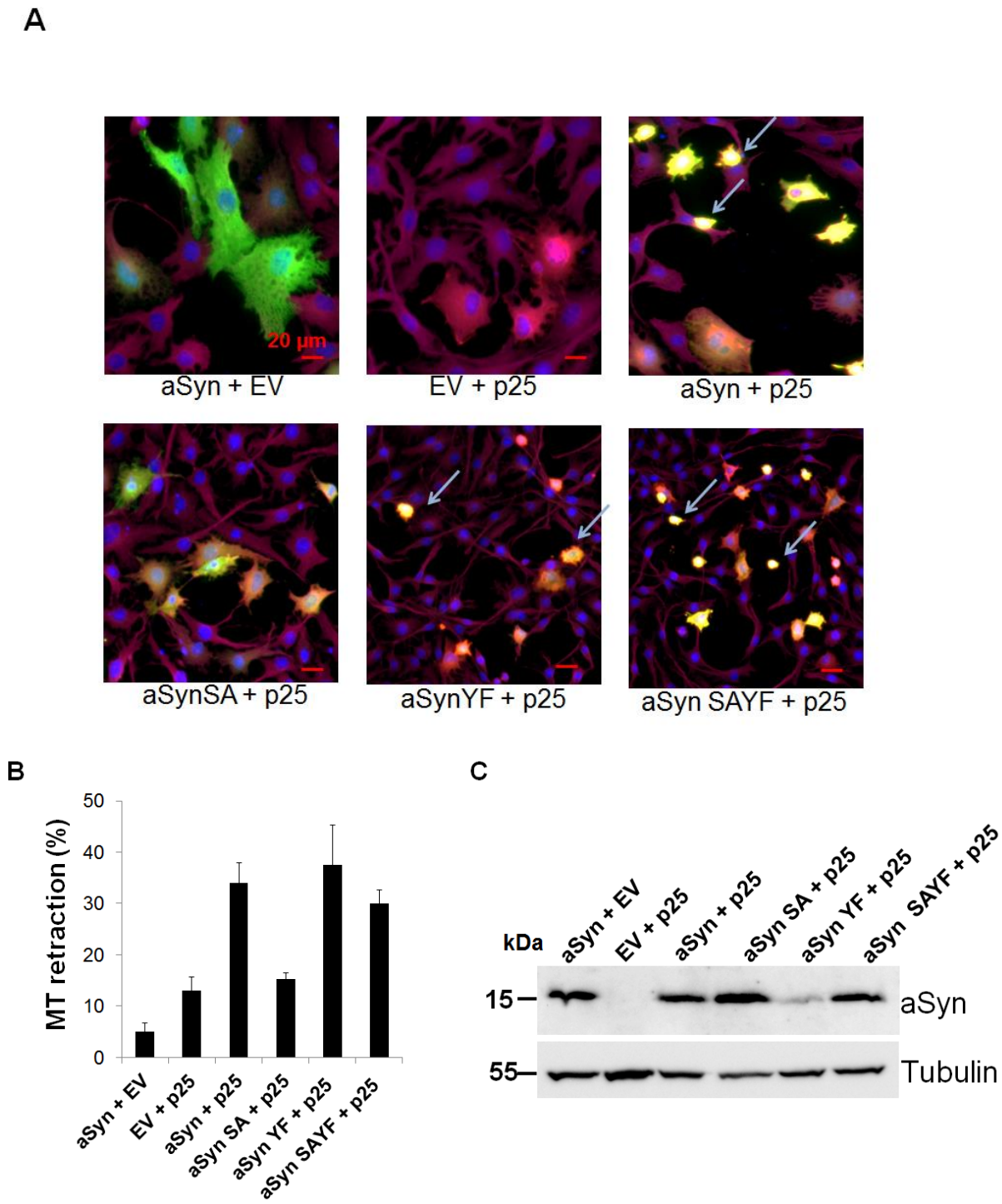
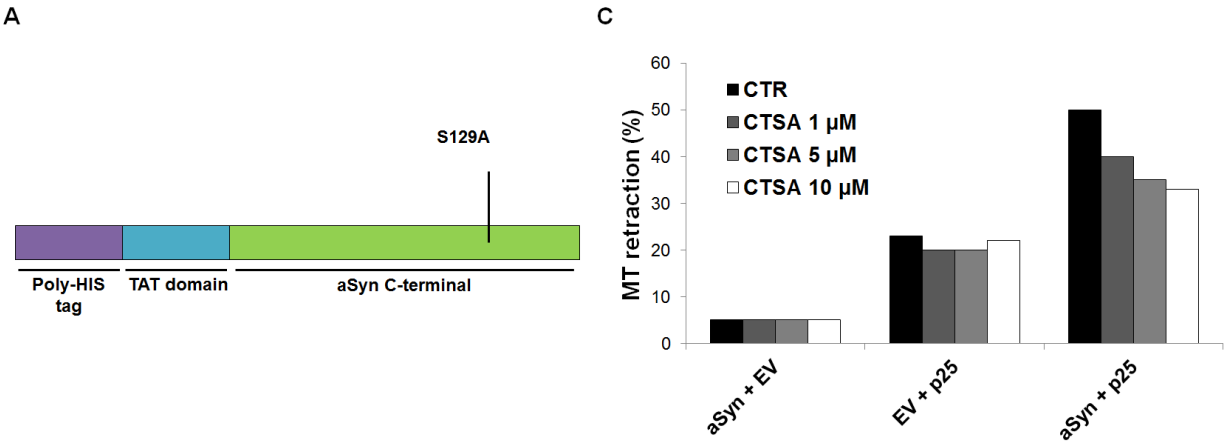


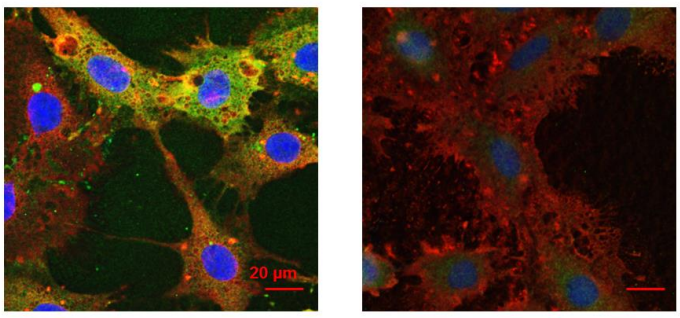
Figure 18: Removal of aSyn C-terminal tyrosine residues abrogates the protective effect of aSyn SA mutation. A. OLN WT cells transiently transfected with aSyn+ EV (empty vector) or p25, aSynSA + p25, aSynYF + p25, aSynSAYF + p25 and subjected to immunofluorescence microscopy using anti aSyn (green), alpha-tubulin (purple) and anti-p25 (red) antibodies. The MT retraction from processes to the perinuclear region upon co-expression of p25 and aSyn is evident (arrows). Note the absence of MT retraction in aSyn + EV and EV + p25 transfected OLN WT cells. **B.** Cellular degeneration is quantified as percentage of aSyn and p25 transfected cells presenting MT retraction in OLN WT cells transiently transfected with aSyn+ EV (empty vector) or p25, aSynSA + p25,

aSynYF + p25 and aSynSA-YF + p25. Bars represent the mean ± S.D **C.** OLN WT cells transiently transfected with aSyn + EV (empty vector) or p25, aSynSA + p25, aSynYF + p25 and aSynSA-YF + p25 were immunoblotted 24 h post transfection using antibodies against total aSyn and tubulin. All data presented are representative of at least three independent experiments.

To test if the unfolded C-terminal of aSyn could be involved in the phenotype observed (independently from the N-terminal region), we developed a strategy to treat cells with a cell-penetrating peptide mirroring this domain. The novel designed peptide was named CTSA (Fig. 19A). CTSA corresponds to the C-terminal domain of aSyn bearing an aminoacid substitution in position 129, where a serine residue is mutated to an alanine residue. It is fused to a TAT domain to favor cell penetration and a hexa-histidine tag to allow for affinity purification (Fig 19A). The peptide was designed to mirror the conditions in which the MT retraction is rescued; which is when aSynSA is co-expressed with p25.



B



OLN WT cells treated with CTSA

OLN WT cells vehicle treated

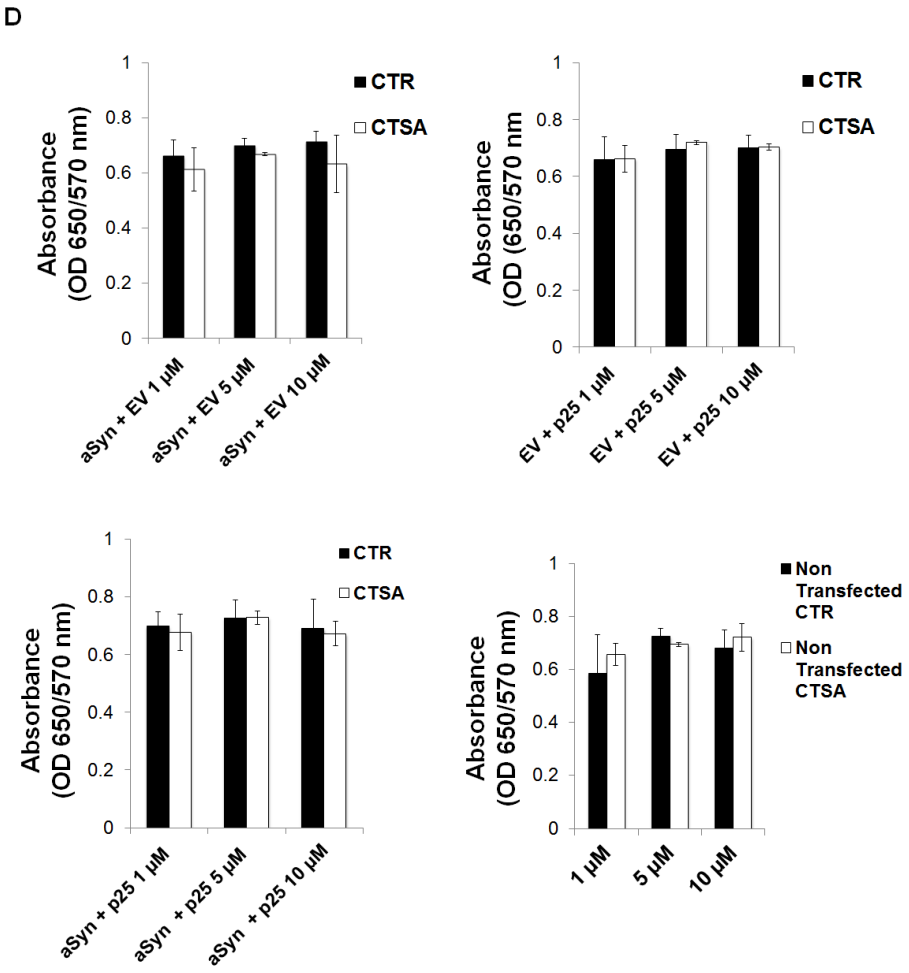


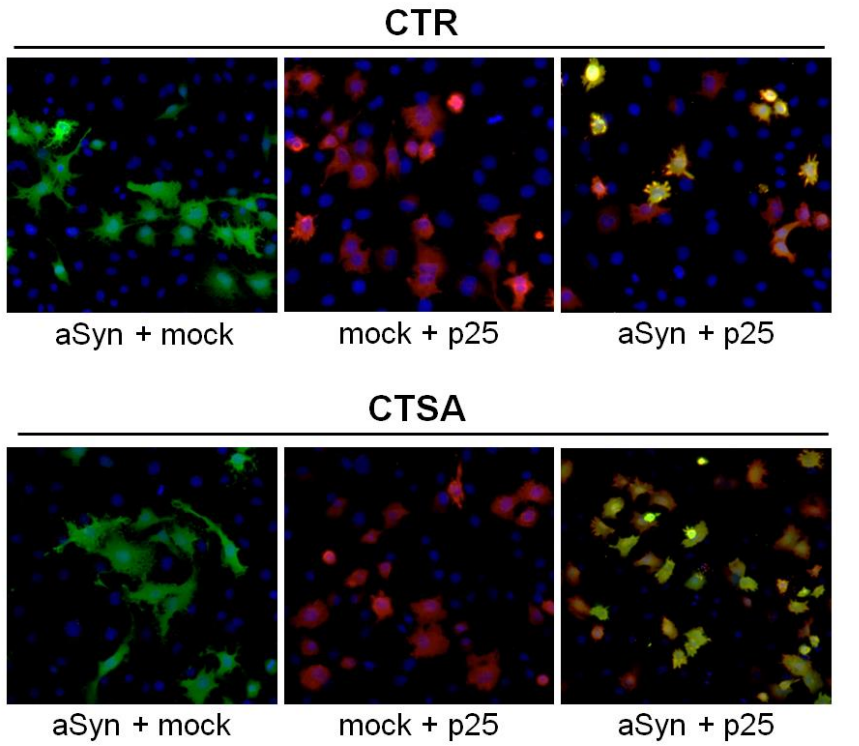
Figure 19. Effects of a cell penetrating peptide mirroring aSyn C-terminal. A. CTSA is a newly designed peptide corresponding to the C-terminal domain of aSyn. The peptide corresponds to the last 40 aminoacid of aSyn in which a serine in position 129 is substitute to an alanine (S129A). Additionally, in the N-terminal were incorporated a TAT domain and a poly-HIS tag. **B.** Cell permeability was tested in OLN WT cells 30 minutes after adding the peptide (5 µM) in the media, cells were fixed and stained for aSyn using ASY-1 antibody (green). Confocal microscopy shows a clear staining only in cells treated with the peptide. **C)** The effect of CTSA or vehicle control (CTR) was tested on MT retraction in the OLN aSyn + p25 model for three different concentrations, respectively 1, 5 and 10 µM. At 5 µM a marked decrease on MT retraction is already visible. Cellular degeneration is quantified as percentage of aSyn and p25 transfected cells presenting MT retraction. The experimental conditions were performed once **D.** Possible toxic effect of CTSA peptide or vehicle control (CTR) was tested in non-transfected and transfected cells at three different concentrations (1, 5 and 10 µM) by MTT assay. No toxicity was detected at any of the indicated concentrations. MTT assay was performed and quantified as described in Materials and Methods. Bars represent the mean ± S.D. All data are representative of at least three independent experiments, unless otherwise stated.

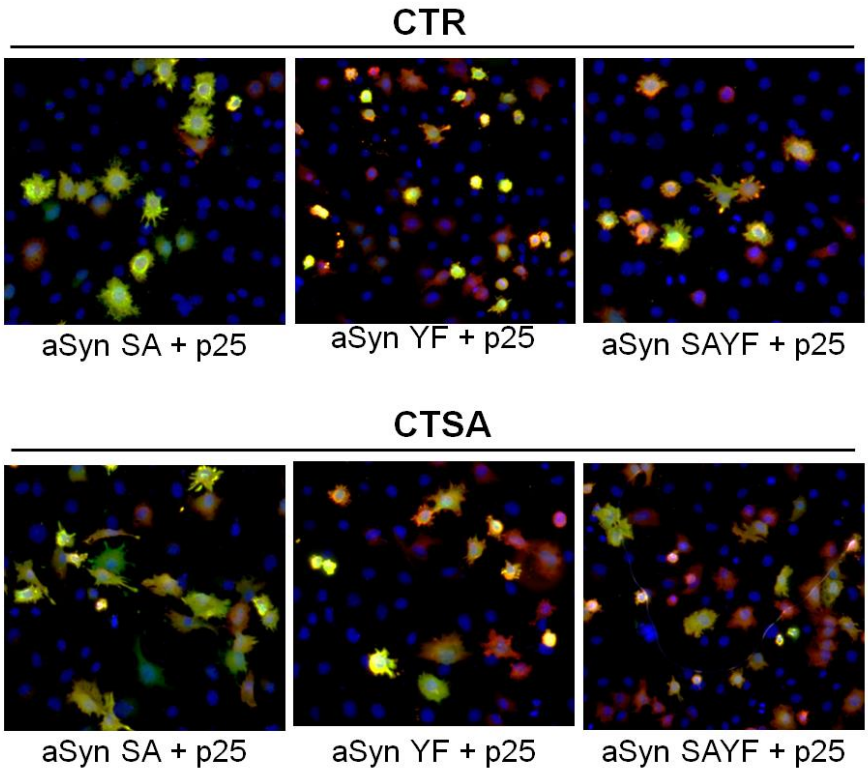
CTSA peptide is able to permeabilize the cells within 30 minutes after its addition to the culture media (Fig. 19B). To determine the optimal working concentration, we tested three

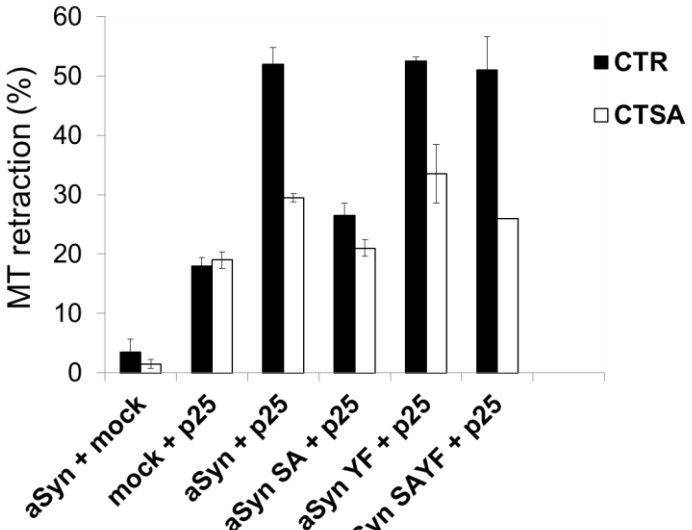
different peptide concentrations: 1, 5 and 10 μM. We then evaluated the effect on MT retraction in oligodendroglial cells expressing aSyn alone, p25 alone and aSyn together with p25, by immunocytochemistry; and the potential toxicity of the peptide by MTT assay (Fig. 19C). Both at 5 and 10 μM it was evident that CTSA is able to decrease the percentage of cells retracting their microtubules when co-expressing aSyn and p25 and no toxicity was detected both in transfected and non-transfected conditions (Fig. 19D). Since we already detected a significant decrease in MT retraction at 5 μM, then we selected this concentration for the study. We added CTSA 4 h post transfection and we performed immunostaining analysis 24 h post transfection (Fig. 20A). The data showed that CTSA is able to reduce the p25 dependent MT retraction in the presence of aSyn WT and aSynSAYF, but it does not further rescue the phenotype of the non-toxic aSynSA mutant (Fig. 20A). Next, we assessed protein expression and phosphorylation levels on Ser-129 by immunoblotting (Fig. 20B). No major differences were detected between control and treated samples. Although CTSA is also able to decrease MT retraction in presence of the aSynYF mutant, the low protein expression levels of this mutant do not allow any conclusions.

Conclusively, the non-phosphorylated Ser-129 aSyn C-terminal can independently of the rest of the aSyn molecule attenuate the toxicity of aSyn and p25 co-expression in our oligodendroglial model of MSA.

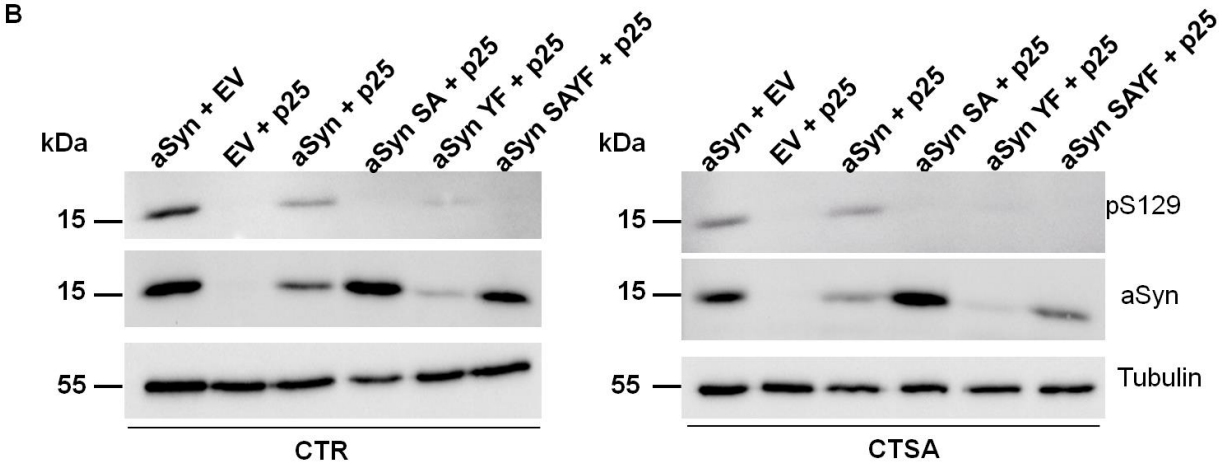
A


CTR
aSyn + mock
mock + p25
aSyn + p25
CTSA
aSyn + mock
mock + p25
aSyn + p25


CTR
aSyn SA + p25
aSyn YF + p25
aSyn SAYF + p25
CTSA
aSyn SA + p25
aSyn YF + p25
aSyn SAYF + p25


MT retraction (%)
60
50
40
30
20
10
0
CTR
CTSA
aSyn + mock
mock + p25
aSyn + p25
aSyn SA + p25
aSyn YF + p25
aSyn SAYF + p25

B


kDa
aSyn + EV
EV + p25
aSyn + p25
aSyn SA + p25
aSyn YF + p25
aSyn SAYF + p25
15
15
55
CTR
kDa
15
15
55
pS129
aSyn
Tubulin
CTSA

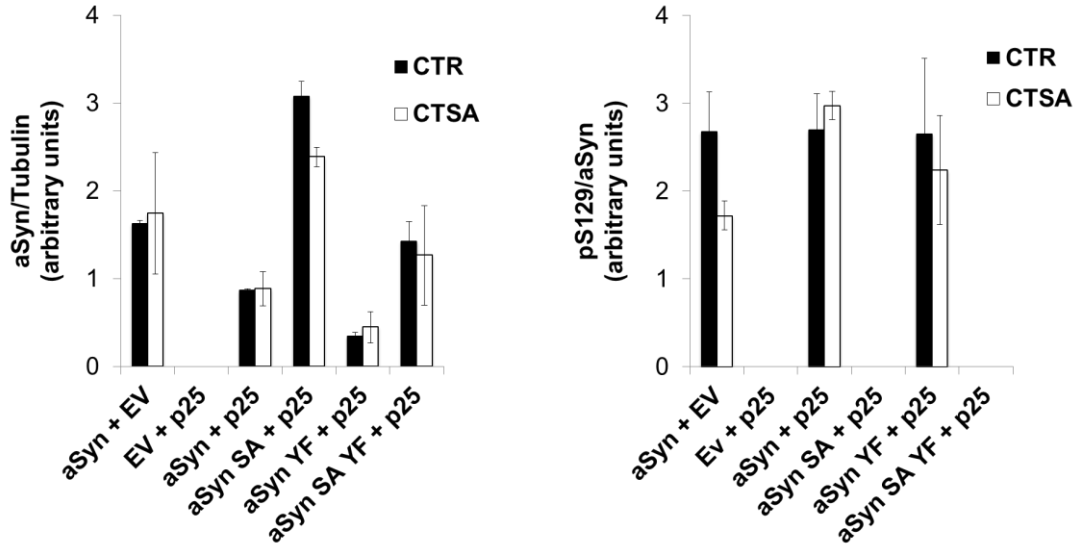
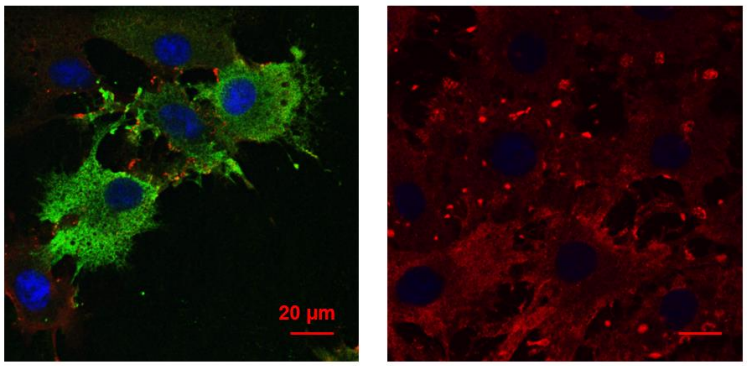

aSyn/Tubulin (arbitrary units)
4
3
2
1
0
CTR
CTSA
aSyn + EV
EV + p25
aSyn + p25
aSyn SA + p25
aSyn YF + p25
aSyn SA YF + p25
pS129/aSyn (arbitrary units)
4
3
2
1
0
CTR
CTSA
aSyn + EV
Ev + p25
aSyn + p25
aSyn SA + p25
aSyn YF + p25
aSyn SA YF + p25

Figure 20. CTSA peptide protect both against cell stress elicited by aSyn WT and aSynSAYF. A. OLN WT cells transiently transfected with aSyn+ EV (empty vector) or p25, aSynSA + p25, aSynYF + p25 and aSynSA-YF + p25 in the presence (CTSA) or absence (vehicle control - CTR) of a novel peptide for 24 h and subjected to immunofluorescence microscopy using anti aSyn and anti-p25 antibodies. The MT retraction upon co-expression of p25 and aSyn is evident (arrows) and it is rescued by CTSA peptide when aSyn+p25, aSynYF + p25 and aSynSAYF are co-expressed. On the right cellular degeneration is quantified as percentage of aSyn and p25 transfected cells presenting MT retraction in OLN WT cells transiently transfected with aSyn + EV (empty vector) or p25, aSynSA + p25, aSynYF + p25 and aSynSAYF + p25 in the presence (CTSA) or absence (vehicle treatedl - CTR) of a novel peptide. Bars represent the mean ± S.D. **B.** OLN WT cells transiently transfected with a aSyn + EV (empty vector) or p25, aSynSA + p25, aSynYF + p25 and aSynSAYF + p25 were extracted in RIPA buffer (see materials and methods) 24 h after transfection and analyzed by immunoblotting, in the presence (CTSA) or absence (vehicle treated - CTR) of a peptide, using antibodies against aSyn phosphorylated on Ser-129, total aSyn and tubulin. Densitometric analysis of the immunodetection of aSyn Ser-129 phosphorylation levels were normalized for the total amount of aSyn (bars represent mean ± S.D), while aSyn total levels were normalized for tubulin levels (loading control). All data presented are representative of three independent experiments.

To further characterize if the effect of the CTSA peptide on MT retraction was dependent on the SA substitution, we produced a control peptide representing the native C-terminal of aSyn, without the SA exchange, and named CT. Similarly to the previous experiments, we tested a concentration of 5 µM on MT retraction, toxicity and permeability (Fig. 21). The peptide behaved like the CTSA peptide by being able to permeabilize the cells within 30 minutes (Fig. 21A), and did not display toxicity to the cells as shown by MTT assay (Fig. 21A). Remarkably it did not affect MT retraction (Fig. 21B). These results demonstrate the beneficial properties of the CTSA peptide and suggest -by analogy to full length aSyn- that this could be due to the removal of the serine 129 phosphorylation residue. In fact, the presence in the CT peptide of solely tyrosine residues as substrate for endogenous kinases is insufficient to protect the cells when Ser-129 can also be phosphorylated.

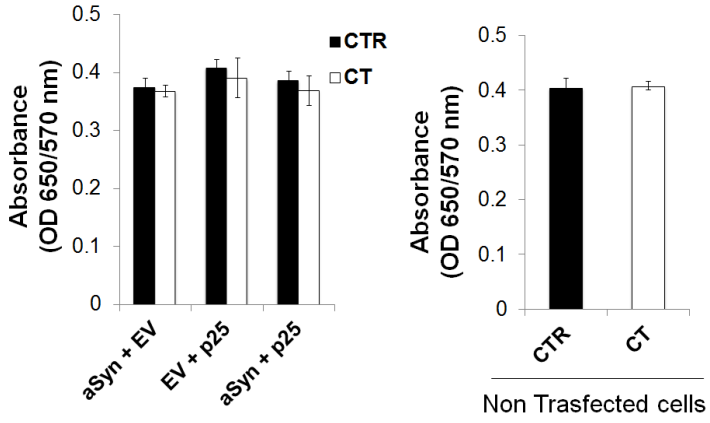
A



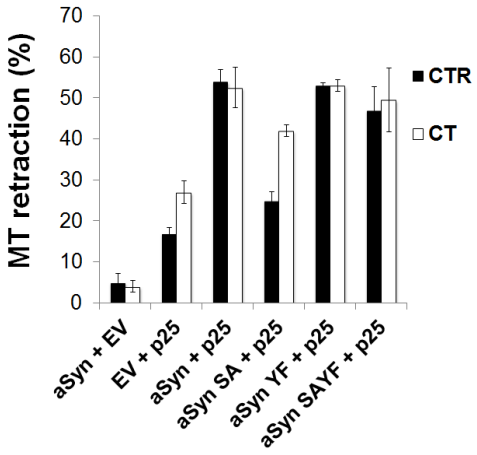
OLN WT cells treated with CT

OLN WT cells vehicle treated

B



C



C

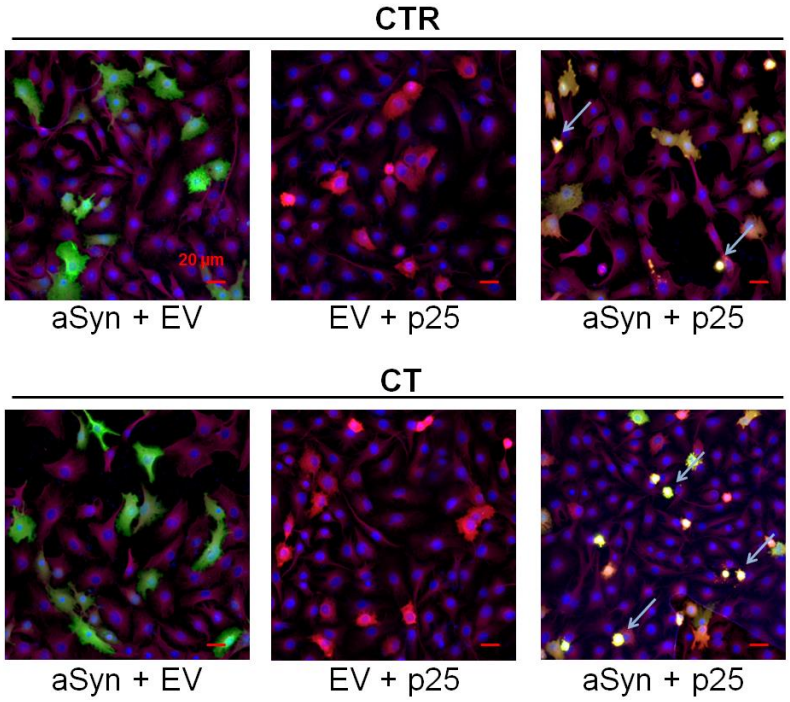


Figure 21. CT, a control peptide, demonstrates the beneficial properties of the CTSA peptide. A. CT is a peptide corresponding to the C-terminal domain of aSyn and therefore identical to the CTSA peptide except without the serine to alanine exchange at residue S129 of the full length aSyn protein (see Fig. 7). Cell permeability was tested in OLN WT cells 30 minutes after adding the peptide (5 µM) in the media. Cells were fixed and stained for aSyn using ASY-1 antibody (green). Confocal microscopy shows staining only in cells treated with the peptide. **B.** No toxicity was demonstrated in cells treated with 5µM of CT peptide or vehicle treated controls (CTR) by MTT assay. MTT assay was performed and quantified as described in materials and methods section. Bars represent the mean ± S.D **C.** The effect of CT on MT was tested at 5µM by immunofluorescence. CT peptide did not change the MT phenotype of cell co-expressing p25 and the aSyn WT, aSyn YF or aSyn SAYF mutants in OLN cells. On the right panel cellular degeneration is expressed as percentage of aSyn and p25 transfected cells displaying MT retraction. Bars represent the mean ± S.D All data are representative of three independent experiments.

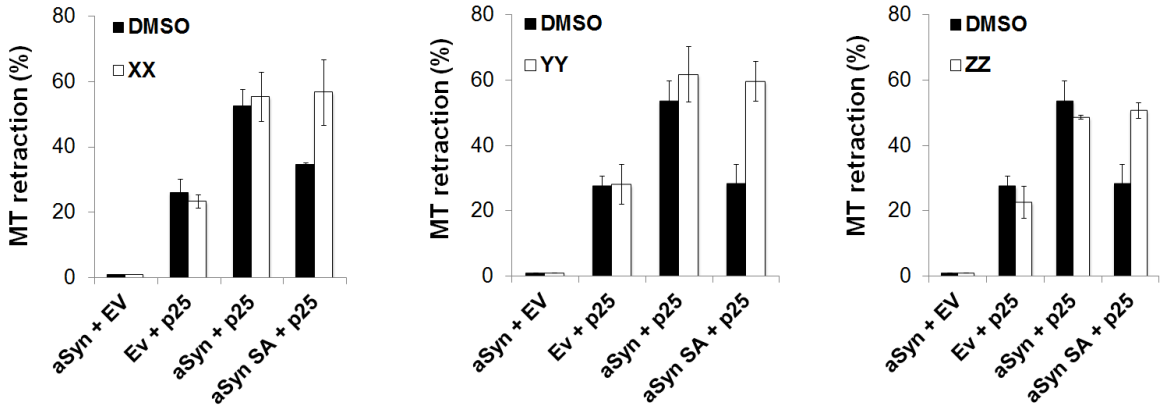
4.5. Effect of tyrosine kinases inhibitors on MT retraction

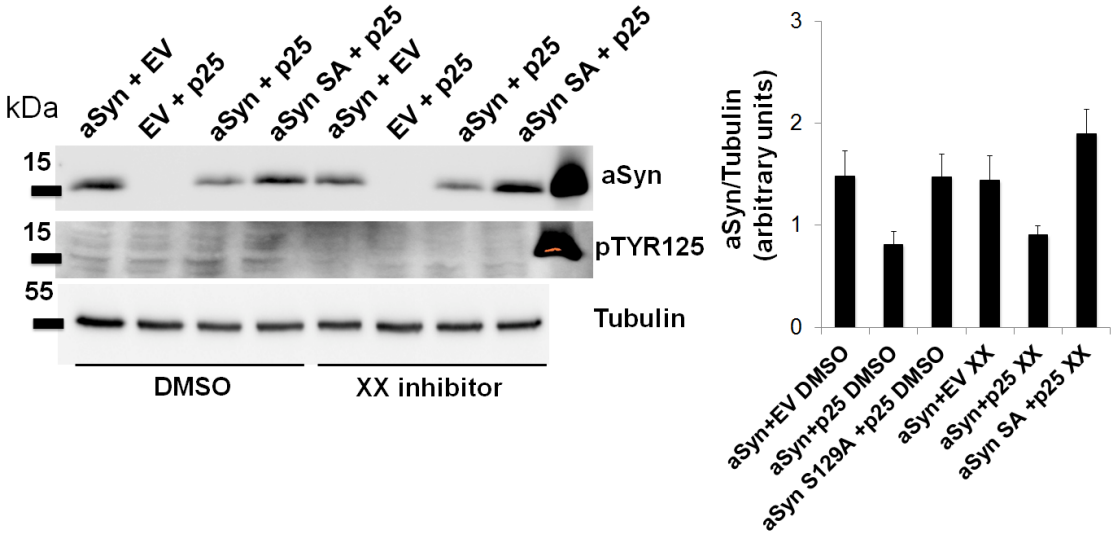
The results obtained suggested the involvement of aSyn tyrosine phosphorylation in the oligodendroglial cellular degeneration and, particularly, its potential protective role. To this purpose a former PhD student Louise B. Vesterager at Lundbeck A/S (laboratory of Poul Henning Jensen, Biomedicine Department, Aarhus University) characterized the effect of a library of kinase inhibitors on the oligodendroglial cell model previously described [246]. A high content screening was performed using automated fluorescence microscopy analysis of cells co-expressing aSyn and p25 treated and non-treated with the selected library of kinase inhibitors. Amongst them 13 different tyrosine kinases inhibitors were able to increase the percentage of cells presenting MT retraction and were chosen to be tested in a cell base assay. Kinases inhibitors targeting specifically tyrosine kinases and thus modulating aSyn tyrosine phosphorylation are expected to increase the MT retraction -due to the abrogation of aSyn tyrosine phosphorylation– and the protective mechanism occurring in our OLN model.

OLN WT cells were transfected with aSyn or aSynSA and p25, treated with the inhibitors 4 hours post transfection and immunostained 24 h post transfection. We validated in our assay three tyrosine kinase inhibitors, for confidentiality purposes named XX, YY and ZZ, as able to increase the MT retraction in presence of aSynSA and p25 (Fig. 22). We performed immunoblotting analysis to assess the level of phosphorylation on Tyr-125 (only antibody commercially available) and aSyn expression levels. Data demonstrated that the inhibitors did not affect aSyn expression levels. The levels of phosphorylation on Tyr-125 were not detected probably due to a low sensitivity of our assay or the contribution of other tyrosine residues (Fig. 22A). Immunofluorescence data for one of the kinases validated are shown as example of the phenotype observed in presence of the inhibitor (Fig. 22B).

Overall our results support the hypothesis that tyrosine kinases are involved in aSyn-mediated toxicity in a cellular model of MSA but we were unable to demonstrate whether active tyrosine kinase inhibitors reduced the phosphorylation of Tyr-125.

A


MT retraction (%)
DMSO
XX
YY
ZZ
aSyn + EV
Ev + p25
aSyn + p25
aSyn SA + p25


kDa
aSyn + EV
EV + p25
aSyn + p25
aSyn SA + p25
aSyn
pTYR125
Tubulin
DMSO
XX inhibitor
aSyn/Tubulin (arbitrary units)
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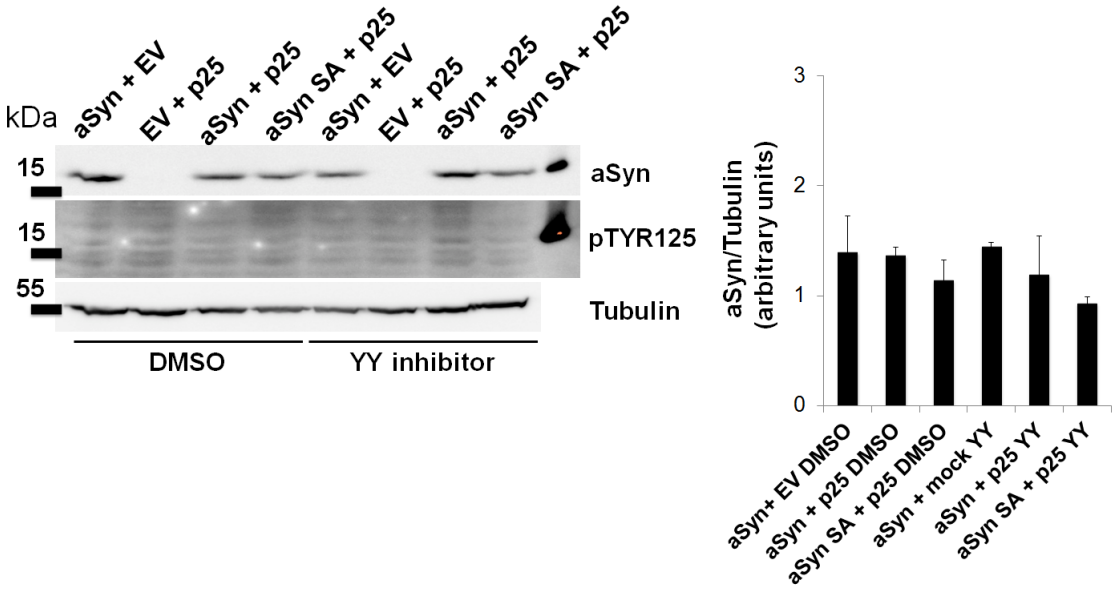
aSyn+p25 DMSO
aSyn S129A +p25 DMSO
aSyn+EV XX
aSyn+p25 XX
aSyn SA +p25 XX


kDa
aSyn + EV
EV + p25
aSyn + p25
aSyn SA + p25
aSyn
pTYR125
Tubulin
DMSO
YY inhibitor
aSyn/Tubulin (arbitrary units)
aSyn+ EV DMSO
aSyn + p25 DMSO
aSyn SA + p25 DMSO
aSyn + mock YY
aSyn + p25 YY
aSyn SA + p25 YY

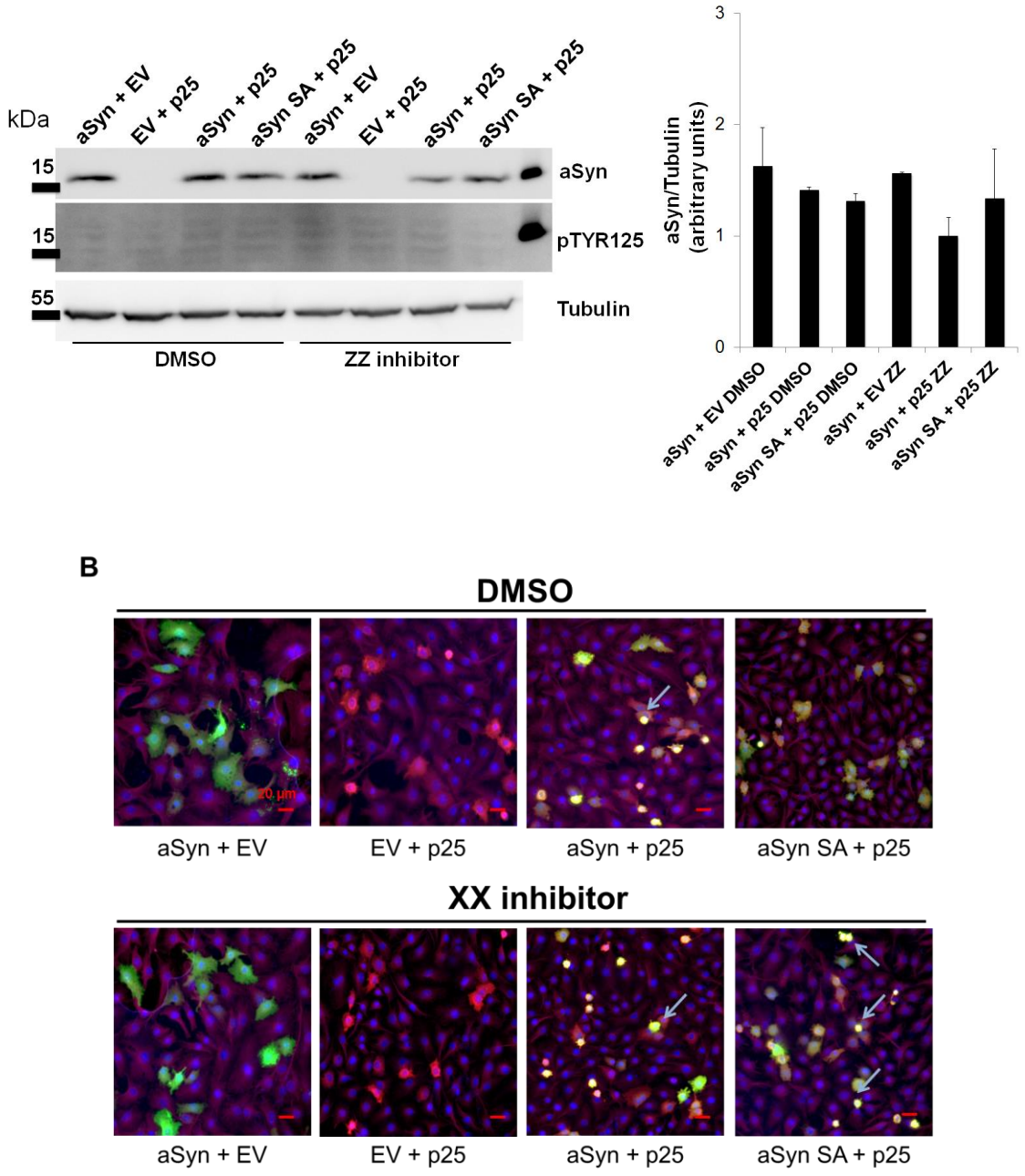


Figure 22. Tyrosine kinase inhibitors increase Ser-129 phosphorylation-dependent MT retraction in an OLN model of MSA. A. On the left panel, cellular degeneration is quantified as percentage of aSyn and p25 transfected cells presenting MT retraction in OLN WT cells transiently transfected with aSyn + EV (empty vector) or p25, and aSynSA + p25, in the presence (XX or or YY or ZZ) or absence (vehicle treatment- DMSO) of a kinase inhibitor. Bars represent the mean ± S.D. On the central panel, OLN WT cells transiently transfected with aSyn + EV (empty vector) or p25, and aSynSA + p25 were extracted in RIPA buffer (see materials and methods section) and immunoblotted 24 h post transfection, in the presence (XX, YY, ZZ) or absence (DMSO) of a kinase inhibitor, using antibodies against aSyn phosphorylated on Tyr-125 and total aSyn. On the right panels, densitometric analyses of the

immunodetection of Tyr-125 phosphorylation levels (pTYR125) were normalized for the total amount of aSyn (mean ± S.D.). **B.** Immunofluorescence of OLN WT cells transiently transfected with aSyn + EV (empty vector) or p25, and aSynSA + p25 were 24 h post transfection, in the presence (XX) or absence (vehicle treatment-DMSO) of a kinase inhibitor, using antibodies against aSyn (green), p25 (red) and tubulin (purple). All data presented are representative of three independent experiments.

5. Discussion

The deposition of misfolded aSyn in the form of proteinaceous inclusions is the hallmark of a number of related neurodegenerative conditions collectively known as synucleinopathies [487]. Within these inclusions, aSyn is known to be post-translationally modified. The modifications include truncation, oxidation, ubiquitination, and phosphorylation, and are thought to affect the conformational and functional state of the protein [316, 319, 344, 375]. Interestingly, 90% of aSyn is phosphorylated on Ser-129 in LBs [9], while only 5% of aSyn is phosphorylated under physiological conditions. Studies conducted until now identified PLK2 and PLK3 as the most efficient kinases phosphorylating aSyn [334]; data in plk2-/- transgenic mouse showed a strong decrease in aSyn phosphorylation on Ser-129 [330]; and the levels of PLK2 increase in AD and Lewy Body disease patients brains [331]. What still remains elusive is the actual role of Ser-129 phosphorylation on aSyn aggregation and its contribution to the pathogenesis of PD.

The present study took advantage of established yeast and mammalian cell models to investigate the role of Ser-129 phosphorylation in the formation of aSyn inclusions ad toxicity. PLK2 and PLK3 were able to phosphorylate aSyn in yeast, but only PLK2 increased aSyn cytotoxicity and the formation of intracellular inclusions. Likewise, a kinase dead PLK2 mutant (PLK2DM) -which was unable to increase aSyn phosphorylation levels on Ser-129- increased the number of cells presenting aSyn foci and cell death, as the active form of the kinase. Additionally, although PLK3 lead to higher levels of Ser-129 phosphorylation than PLK2; it did not increase aSyn cytotoxicity or inclusion formation in yeast. Assessment of the biochemical state of aSyn using ultracentrifugation in sucrose gradients revealed an increase in oligomeric species with higher molecular weight, when PLK2, PLK2DM or PLK3 were co-expressed. Nonetheless, PLK2 and PLK2DM induced a different pattern of distribution of aSyn species, confirming a specific role for PLK2 in aSyn foci formation and toxicity in yeast cells.

Altogether, these data suggest that PLK2 increases aSyn foci formation independently of the levels of phosphorylation of aSyn on Ser-129 and that the action of PLK2 on aSyn might involve additional effects on targets other than just aSyn, or on other aSyn phosphorylation sites.

The results obtained in yeast were then validated in human cells. The BiFC assay enabled us to investigate the role of PLK2 in a model of aSyn dimerization/oligomerization [488]. We observed the formation of aSyn cytoplasmic inclusions in cells co-expressing PLK2. Using BI2536, a known inhibitor of PLK2 kinase activity, we assessed the role of aSyn Ser-129 phosphorylation on the formation of aSyn inclusions. BI2526 reduced aSyn phosphorylation and affected PLK2-mediated aSyn inclusion formation.

To further explore the effect of PLK2 on aSyn misfolding an aggregation model based on the co-expression of a C-terminally modified form of aSyn (SynT) and synphilin-1 was used in CAD cells

[13, 183]. Results demonstrated that PLK1 was not able to increase the levels of aSyn Ser-129 phosphorylation but affected the levels of synphilin-1 and may have consequently influenced the formation of aSyn larger inclusions. On the other hand, PLK2 was found to significantly increase aSyn Ser-129 phosphorylation and inclusion formation, in agreement with the results obtained in yeast. As PLK2 does not alter the levels of synphilin-1 and induces the formation of aSyn inclusions, these results further imply that the generation of aSyn inclusions requires specific factors, further validating the effect of PLK2 in these cellular models of aSyn aggregates formation. Despite the ability of PLK3 to increase the phosphorylation levels of aSyn, we could not further investigate its role on aSyn aggregation, as it drastically reduced the levels of aSyn, suggesting it might promote its degradation. The biochemical nature of the aSyn inclusions formed in the presence of PLK2 was also assessed and, in agreement with the data obtained in yeast cells, PLK2 caused an overall increase in larger Syn species.

Here, we provide evidence that PLK2 may be involved in the formation of aSyn aggregates in a way that is not solely dependent on its ability to phosphorylate aSyn on Ser-129. Recent studies have showed that aSyn may directly impair PLK2 activity and, consequently, inhibit the signaling pathway regulated by PLK2, namely p38 MAPK [489]. Furthermore, PLK2 has been also involved in the inhibition of the mTOR pathway -in a tumor growth context- through the interaction with tubero sclerosis complex 1 (TSC1) [490]. Interestingly, mTOR pathway is also a known regulator of the autophagy degradation system that, together with the ubiquitin-proteasome system, is known to be responsible for the clearance of aSyn [386]. A very recent paper has proved evidence for the latter hypothesis in a rat genetic model over-expressing human aSyn and PLK2. The data demonstrated that PLK2 mediated aSyn degradation through both phosphorylation at Ser-129 and formation of a protein complex with aSyn [491]. These findings demonstrate that PLK2 may influence aSyn turnover, but more importantly, the role played by PLK2 expression in aSyn pathobiology as fundamental as its capacity to phosphorylate aSyn on Ser-129. This concept as demonstrated by our studies obtained combining yeast and mammalian cell models, provide novel experimental support in favor of PLK2 as a target for therapeutic intervention in synucleinopathies.

We then performed a functional screening in the yeast *Saccharomyces cerevisiae* to identify novel kinases able to modulate aSyn pathobiology. Yeast has been widely and positively used to characterize novel genes involved in the neurodegeneration process [445]; therefore it was exploited in the present study. Within the yeast kinome, kinases homologous to human ones were identify and the most promising ones -based on literature searching and tissue expression- were characterized. Two kinases showed a phenotype aSyn dependent: Atg1 and Sky1 decreased yeast cell growth; with Sky1 also able to decrease aSyn foci formation. Conversely, they all failed to modulate aSyn phosphorylation on Ser-129, although the characterization of other phosphorylation sites remains fundamental.

The most promising appears to be Atg1, a kinase involved in autophagy, a cellular process whose dysfunction has been broadly described in several neurodegenerative disorders [492]. Autophagy is mainly dependent on nutrient condition; upon induction, by starvation or treatment with rapamycin (TOR inhibitor), Atg13 is quickly dephosphorylated, a state that promotes its interaction with Atg1 initiating the autophagic pathway [493]. Importantly, Atg1 is a highly conserved gene, its human homologous Ulk1 has been well characterized and it is known to play a similar function to the yeast one [494].

In mammalian cell models Atg1 kinase function is believed to be fundamental in the induction of autophagy [495], its over-expression has been used as model to induce autophagy and it has been correlated with impaired cell growth and death [483]. Interestingly, in our yeast model we detected a decrease in cell growth in cells co-expressing aSyn and Atg1, suggesting the dependence of our phenotype on aSyn expression. Furhermore, the yeast autophagosome/vacuole system has been shown as primarily involved in aSyn clearance [446], supporting our finding on Atg1 contribution. Additional experiments will verify if autophagy and aSyn clearing are indeed induced in our model since we would expect a decrease in aSyn foci bearing cells, which was not observed. Conversely, it was shown that the seeding of larger aggregate in cell lines and primary neuronal cultures inhibits aSyn degradation, in particularly impeding autophagosome clearance and increasing cell death [496], sustaining also our findings; therefore further data will aim to evaluate aSyn specied formed when co-expressed with Atg1 and the effect of Atg1 gene deletion on yeast cells growth.

In this scenario, Atg1 is a novel potential kinase involved in aSyn pathobiology and may represent an additional step toward elucidating the molecular mechanisms of synucleinopathies.

In addition to serine residues, aSyn can be phosphorylated at tyrosine residues Tyr-125, Tyr-133, and Tyr-136 *in vitro*. However, only phosphorylation at Tyr-125 has been detected *in vivo* [360, 367, 370, 371]. Recent data demonstrated that Tyr-125 phosphorylation diminishes during the normal aging process in both humans and flies. Furthermore, in flies' models of PD Tyr-125 phosphorylation can modulate Ser-129 phosphorylation mediated toxicity, suggesting a potential cross-talk between these two modifications that may play a central role in aSyn function and may be lost if looking at each modification independently. aSynYF mutant flies demonstrated a decreased in climbing ability compared with aSyn WT flies; whereas increasing physiologically the levels of Tyr-125 phosphorylation decreased oligomer formation and rescued aSyn cytotoxicity, suggesting a neuroprotective function for Tyr-125 phosphorylation [371].

Unfortunately, little is known about the functional consequences of tyrosine phosphorylation, and whether tyrosine and serine phosphorylation might regulate each other. Thus, to characterize the role of tyrosine phosphorylation on aSyn toxicity, we decided to take advantage of an established oligodendroglial cell model of MSA. In this model (OLN) co-expression of aSyn and p25 induces cell degeneration reflected by microtubule (MT) retraction and activation of apoptosis. Remarkably,

this phenotype can be rescue by a non-phosphorylated aSyn at Ser-129 (SA) [246]. We therefore decided to study the cells displaying MT retraction in presence of several aSyn phosphorylation mutants: aSynSA, aSynSAYF and aSynYF. A decrease in MT retraction was evident only in the presence of aSynSA, as previously described [246], but also suggesting that tyrosine residues need to be available to rescue cell degeneration; and, interestingly, the possibility of a cooperative role for Ser-129 and Tyr-123, 133 and 136 in aSyn mediated MT retraction.

Considering the results obtained, we designed a cell penetrable peptide able to mimic the protective mechanism represented by aSynSA mutant. We then produced the CTSA peptide and tested in the OLN model. Interestingly, CTSA was able to rescue MT retraction in presence of aSyn WT and aSynSAYF, demonstrating that supplementing the intracellular compartment with aSyn C-terminal domain inhibits MT retraction. This may be due to a decrease binding of p25 to aSyn -indeed p25 promotes aSyn aggregation process [216]- or to the activation of signalling pathways mediated by the presence of further phosphorylable tyrosine residues. Tyrosine phosphorylation is indeed known as a common signalling process that induces cell growth and differentiation [497]. It was not possible to analyze the effect of CTSA on aSynYF mutant due to the low protein expression levels of this mutant; we hypothesized that this mutant generates higher insoluble aSyn species that necessitate of different extraction detergent. Additional studies will be required to investigate the aSynYF expression behavior.

We used a control peptide mirroring the C-terminal of aSyn to verify if the effect produced was related to the absence of a phosphorylable serine in position 129. Remarkably, this peptide failed to reduce MT retraction in all the conditions previously tested. This suggests that adding a peptide consisting of the C-terminal part of aSyn is insufficient to promote protection, despite the presence of phosphorylable tyrosine residues. Further studies will verify this hypothesis using other peptides reproducing the absence of phosphorylable tyrosine (YF) and the absence of phosphorylable Ser-129 and tyrosine residues (SAYF), respectively.

Altogether, these data corroborate a protective role for tyrosine phosphorylation in aSyn pathobiology, suggesting tyrosine phosphorylation as modulator of aSyn toxicity dependent on the phosphorylation status of Ser-129. The tyrosine-phenylalanine mutation is very minor and only removes a hydroxyl group from tyrosine; thus it is reasonable to assume that the effect produced is due to the absent phosphorylation of the hydroxyl group.

In parallel, we studied a panel of 13 kinases inhibitors and we validated 5 of them in the OLN model here already extensively described [246]. The effect of these kinase inhibitors was tested on MT retraction, tyrosine phosphorylation and toxicity. All the inhibitors increased MT retraction in presence of both aSyn and p25 showing an effect aSyn dependent. We performed immunoblotting analysis to characterize if the inhibitors were affecting aSyn expression levels and, most importantly, tyrosine 125 phosphorylation levels. The expression levels were unaffected, but unfortunately we did not detect any phosphorylation signal; this may be due to a low sensitivity of

the assay used or the involvement of other tyrosine residues. It is indeed known that tyrosine phosphorylation is an extremely rapid cellular process that may be difficult to characterize by classic immunoblotting technique [498]. Further studies will confirm this hypothesis adopting immunoprecipitation analysis and use of phosphatase inhibitors to stabilize tyrosine phosphorylation and thus enhance its detection.

In summary, these studies corroborate a tight link between tyrosine and serine phosphorylation in aSyn misfolding behavior, showing a protective mechanism associated with tyrosine phosphorylation and characterizing novel kinases as potential target for therapeutic purposes. Furthermore, we identified a cross-talk between Ser-129 and tyrosine phosphorylation sites, unraveling the necessity to study in concert these post-translational modifications and demonstrating that further experimental approaches have to take in consideration both these phosphorylation residues to understand aSyn dysfunction in synucleinopathies and to try to eliminate data discrepancies among different model organisms, which might result from differential post translational processes.

6. General conclusions and future perspectives

Phosphorylation is the main post-translational modification used by any cell type to initiate, modulate or turn-off a signaling cascade [499]. Phosphorylation modulates protein function and activity without the costly necessity of producing new proteins; unfortunately this process is often found altered in several human diseases such as neurodegenerative disorders. Here, abnormal phosphorylation contributes to protein misfolding, aggregation and ultimately to the pathogenesis of the disorder.

aSyn is amongst those proteins that is anomaly phosphorylated in synucleinopathies, and numerous researchers are trying to understand how this post-translational modification goes awry. In this scenario, looking at the kinases and phosphatases responsible for aSyn phosphorylation can explain how this modification is acting upon aSyn misfolding behavior opening up new targets for therapeutic intervention.

In this study, we explored the effect of known kinases able to phosphorylate aSyn on Ser-129 on toxicity and inclusion formation in established yeast and mammalian cell models, and we performed a functional screening in yeast to identify novel kinases involved in aSyn pathobiology. Similarly, we characterized the role of tyrosine phosphorylation in terms of toxicity and inclusion formation in an oligodendroglial cell model and we assessed novel potential tyrosine kinases inhibitors.

Several studies have substantiated the role of PLK2 in aSyn phosphorylation on Ser-129, but failed to understand the link with PD pathogenesis. Our results, combining yeast and mammalian cell models, provide novel experimental support in favour of PLK2, but most importantly, demonstrate that PLK2 involvement in PD pathogenesis goes beyond its ability to phosphorylate aSyn but reside in its mere expression. We showed that PLK2 co-expression induced the formation of more inclusions in yeast and larger in cell lines, whose toxicity was proven in yeast but remains to be addressed in cells. Remarkably, novel recent studies have pointed to a PLK2-mediated aSyn clearance by autophagy, which resulted protective to cells [498]. In the light of these results, we can hypothize that the step between PLK2 being protective or toxic may reside in aSyn species formation, with soluble oligomers more prone to be cleared and larger aggregates leading to cell death. Further data are necessary to elucidate which precise PLK2 signaling cascades interfere with aSyn inclusion formation in order to implement it as novel therapeutic intervention in synucleinopathies.

Our yeast functional screening allowed the characterization of new kinases involved in aSyn pathobiology, extending and corroborating our view on the molecular mechanisms involved in aSyn toxicity. Here we provided evidence of Atg1, a fundamental kinase within the autophagy signaling cascade, as novel modulator of aSyn pathobiology, unlocking a captivating scenario into the role of

aSyn toxicity. Once more autophagy may play a role between the pathogenic and functional state of aSyn thus proving fundamental to explore Atg1 involvment in cell models of PD and synucleinopathies.

In this scenario, we also demonstrated that we cannot exclude other aSyn phosphorylation sites known to be associated with the pathogenesis of these disorders, to understand aSyn dysfunction. In the only work from PD human brain samples; the phosphorylation levels of Tyr-125 decreased with age and with Lewy body pathology, corroborating the involvement of Tyr-125 phosphorylation in the pathogenesis and, notably, a potential protective role [371]. Our investigation validate this mechanism: expression of a non-tyrosine phosphorylable version of aSyn (YF) showed increased cellular toxicity that could be rescued adding extra tyrosine residues in the form of a peptide representing aSyn C-terminal domain non-phosphorylated at Ser-129 reside (CTSA). Although additional data will elucidate the molecular mechanism behind the protective function of the CTSA peptide; our results demonstrate for the first time the role play by an isolated aSyn C-terminal domain on cellular aggregation dependent toxicity. The investigation of tyrosine kinases involved in aSyn tyrosine phosphorylation using selective inhibitors, offered further experimental support to our findings and the possibility to develop novel therapeutic targets for synucleinopathies.

The present study establishes crucial lines of investigation on the role of aSyn phosphorylation in synucleinopathies and validates the use of different model systems in the study of aSyn pathobiology. We validated the implication of Ser-129 phosphorylation in the pathogenesis of the disease, a cooperative function between Ser-129 and tyrosine residues and the essential involvement of aSyn C-terminal domain in aSyn dysfuntion.

All in all, uncovering novel targets in the continuous search for new therapeutic treatments will enable us to move forward towards the development of more effective therapies for devastating disorders of the nervous system, including synucleinopathies, which were the focus of this thesis.

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